Oestrogens and Consequences for the Excurrent Ducts in the Male

Jane Susan Fisher

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Centre for Reproductive Biology
37 Chalmers Street
Edinburgh, EH3 9EW

Declaration

The research described in this thesis is the sole work of the author and is not being submitted in support of another degree or qualification at this university or any other university or institute of learning.

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Abstract

The aim of the work described in this thesis was to identify the sites and potential roles of oestrogen action in the development and function of the male reproductive tract, particularly within the excurrent ducts. The sites of oestrogen receptor-beta (ERβ) immunoexpression were identified within the testis and epididymis of the marmoset monkey from perinatal to adult life and compared to those of oestrogen receptor-alpha (ER-α) and the androgen receptor (AR). One common site of expression, the efferent ducts, was focused on in the remainder of the studies. Neonatal rats were injected on alternate days between days 2 - 12 with DES at 3 doses (10, 1 and 0.1μg/injection) or with ethinyl oestradiol (EE; 10μg/injection) or with high doses of one or other weakly oestrogenic compounds (genistein, octylphenol, bisphenol A or parabens) or the agonist/antagonist Tamoxifen. To establish whether the effects of oestrogen treatment were direct or indirect (i.e. arising from LH/FSH secretion) all parameters investigated were also analysed in rats treated neonatally with a gonadotrophin-releasing hormone antagonist (GnRHa). Animals were investigated at 18, 25, 35 and 75 days (adulthood) of age. Morphology of the excurrent ducts was assessed with each treatment and at each time point. High dose oestrogen treatment (10μg of DES or EE) induced gross distension and overgrowth of the rete testis, though no effects were detected after treatment with weakly oestrogenic compounds. The efferent ducts demonstrated 3 major dose-dependent changes after treatment with DES or EE. (1) the efferent duct lumens were abnormally distended. (2) the normally tall columnar epithelial cells were low cuboidal and lacked much of the apical cytoplasm and brush border; measurement of epithelial cell height confirmed these changes. (3) the water channel protein Aquaporin-1 (AQP-1) which is normally expressed on the apical membrane of non-ciliated cells was greatly reduced in expression after treatment with potent oestrogens. The effects on the efferent ducts were most pronounced at days 18 and 25 and seemed to normalise by day 75 (except for rete testis distension).

The reduction in epithelial cell height was the only parameter which showed a detectable, significant change after exposure to weak oestrogens. None of these changes were detectable in rats treated neonatally with GnRHa. Adult oestrogen receptor knockout (ERKO) mice showed a reduction in AQP-1 immunostaining in the proximal region of the efferent ducts comparable to that seen after high dose DES treatment. In rats treated neonatally with 10μg DES there was a pronounced reduction in immunoexpression of the androgen receptor throughout the testes, rete testis, efferent ducts and epididymis at days 10-25 of age whereas immunoexpression of both oestrogen receptors remained unchanged. A preliminary study was also made of the effects of neonatal oestrogen treatment on ion channel expression within the epididymis. Studies of the cystic fibrosis transmembrane regulator (CFTR) using in situ hybridisation were inconclusive but the immunoexpression of two other ion channel proteins (H^+-ATPase and Na^+/H^+ exchanger), which have a role in lumenal acidification, were studied after neonatal oestrogen treatment. The number of cells expressing the Na^+/H^+ exchanger was unchanged whereas the number of epididymal clear cells expressing the H^+-ATPase channel was significantly reduced after oestrogen treatment. It was unclear whether this was a direct effect of oestrogen treatment as similar, but less severe, effects were detected after GnRHa treatment. These studies suggest that oestrogens play a role in both the development and function of the male reproductive system, especially of the excurrent ducts. It is concluded that physiological levels of oestrogen are required for normal testis growth and spermatogenesis, and that oestrogens are involved in maintaining normal fluid dynamics within the excurrent duct system. Oestrogens appear to play a role in the development of the efferent duct epithelium as both cell morphology and function were altered after exposure to oestrogens. The reduced fluid resorption suggests this function is at least in part modulated by oestrogen. Oestrogen exposure also influenced androgen action as the level of androgen receptor (as detected by immunocytochemistry) was negligible or greatly reduced after neonatal oestrogen treatment. More specific endpoints of oestrogen action have yet to be identified and

research is required to allow a fuller understanding of the roles of oestrogen and to determine whether exposure to environmental or dietary oestrogens pose any risk to human reproductive health.

Abbreviations

5HT	5-hydroxytryptamine
ABP	androgen binding protein
ACE	angiotensin converting enzyme
ACTH	adrencorticotrophic hormone
ANOVA	analysis of variance
APEO	alkylphenol polyethoxylate
AQP-1	aquaporin-1
AR	androgen receptor
AT	apical tubule
AVP	arginine vassopressin
BSA	bovine serum albumen
CF	cationic ferritin
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP28	channel forming integral membrane protein of 28KDa
CIS	carcinoma in situ
DDT	trichloroethane
DES	diethylstilbestrol
DNA	deoxyribose nucleic acid
DHT	dihydrotestosterone
DSP	daily sperm production
EE	ethinyl oestradiol
EDS	ethane dimethyl sulphonate
ER	oestrogen receptor
ERα	oestrogen receptor alpha

ERβ	oestrogen receptor beta
ERE	oestrogen response element
ERKO	oestrogen receptor knock-out
FSH	follicle stimulating hormone
g	gram
GH	growth hormone
GnRH	gonadotrophin releasing hormone
GnRHa	gonadotrophin releasing hormone antagonist
hCG	human chorionic gonadotrophin
HRP	horseradish peroxidase
ICBDMS	international clearinghouse for birth defects monitoring systems
IF	interstitial fluid
KDa	kilodalton
L	litre
LH	luteinizing hormone
MIP	major intrinsic protein of the lens
MIS	Müllerian inhibiting substance
ml	millitre
NBT	nitroblue tetrazolium
NOEL	no observable effect level
NSS	normal swine serum
PBS	phosphate buffered saline
p.c.	post coitum
PCB	polychlorinated biphenol
PRL	prolactin
RAS	renin-angiotensin system
RNA	ribonucleic acid

RTF	rete testis fluid
SD	standard deviation
SHBG	sex hormone binding globulin
STF	seminiferous tubule fluid
TBS	tris buffered saline
TCP	tubular coated pit
TSH	thyroid stimulating hormone

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Chapter 1 Literature Review

1.1 Introduction

Until recently, studies into male reproductive health have been largely based on understanding the physiology of the male reproductive system, including the process of spermatogenesis, and developing novel contraceptive methods to control male fertility. Over the past 8 years, an increasing number of papers in the literature have expressed concerns that human male reproductive health is declining and that the incidence of congenital abnormalities and cancers of the male reproductive tract is increasing. The causes behind these reported increases are not known but one hypothesis that has been promoted in both the scientific community and the media is that exposure to exogenous oestrogen or oestrogen-like compounds (xenoestrogens, environmental oestrogens) may be involved. Studies that have assessed the impact on humans of *in utero* exposure to a potent synthetic oestrogen (diethylstilbestrol) have shown increases in similar disorders in the male offspring.

The role of oestrogen in male reproductive physiology is not well understood and more insight is needed into how this steroid affects normal physiology before assessment of any effects from abnormal exposure can be made with confidence. It is known that the enzyme aromatase, which converts testosterone into oestrogen, is present within the male reproductive tract, as are the protein receptors which bind oestrogen (oestrogen receptors α and β; ER- α and β) and promote oestrogen induced gene transcription.

The experimental work addressed in this thesis was performed to make a contribution to understanding the role of oestrogen in male reproductive physiology and to give some insight into the processes that are affected after exogenous exposure to potent oestrogens and some 'environmental oestrogens'. The literature review aims to give an overview of the physiological control of male reproduction, to explain the structure of the excurrent ducts of the male reproductive tract and to examine the evidence surrounding the current controversies regarding the reported changes to male reproductive health.

1.2 Evidence That Decreasing Sperm Counts have Links with Abnormal Oestrogen Exposure

The hypothesis that factors within the environment that mimic the action of the steroid hormone oestrogen may be having an impact on sperm counts was proposed in the early 1990s. There were an increasing number of reports in the literature that the human sperm count had fallen over the past 50 years. Co-incident with these reports there were studies

suggesting that congenital malformations of the male reproductive tract (hypospadias, cryptorchidism) and testicular germ cell cancer were also increasing. These pathologies struck researchers as very similar to those that had arisen after the administration of a potent synthetic oestrogen, diethylstilbestrol (DES). This compound was administered to pregnant women and subsequently to laboratory animals and was shown to induce reproductive abnormalities in the offspring born to these mothers. There is an ever increasing list of chemicals that are wide-spread in the environment and have been shown to be hormonally active, some acting as oestrogens or antioestrogens and others as anti-androgens. In general however, the binding affinity of these chemicals for the relevant steroid receptor is magnitudes lower than that of the natural ligands.

1.2.1 Declining Sperm Counts

The study that sparked a huge controversy in male reproductive health and highlighted the debate as to whether there had been a genuine decrease in sperm concentration over the past 50 years was published in 1992. This paper by Carlsen *et al.* (1992) was a meta-analysis of the international literature on sperm counts and semen volume from normal men between 1938 and 1990 (Carlsen *et al.*, 1992). The analysis included data from 61 studies on almost 15,000 men and suggested that there had been a decrease in both sperm concentration and semen volume over time. Sperm concentration was reduced from an average of 113 million/ml to 66 million/ml and semen volume was also reduced from 3.40 ml to 2.75ml (Carlsen *et al.*, 1992). The debate as to the validity of the conclusions drawn from the data are still ongoing (Brake and Krause, 1992; Bromwich *et al.*, 1994; Farrow, 1994)(Olsen *et al.*, 1995).

This is not the first analysis of semen quality which has suggested that sperm counts are falling, in fact there were several reports published in the 1970-80s (reviewed by Irvine, 1997). A study from 1974, reported on data from 386 men from Iowa who had their semen analysed prior to vasectomy. The mean sperm count was 48x 10^6/ml and the authors noted that these values were far lower than previous studies of semen quality in the literature from the 1930-50's which reported average sperm counts at 120x10^6 (n=200) (reviewed in Irvine, 1997) 145x 10^6/ml (n=49) (Farris, 1949) and 100.7x 10^6/ml (n=100) (Faulk and Kaufman, 1950). In 1981 a report on semen quality among potential semen donors was published which found that the number of men having acceptable semen quality in 1973 had fallen from 77% to 37% in 1980 using the same selection standards (Leto and Frensilli, 1981). A review of the published data was reported in 1980 and the author observed a negative correlation between later year of publication and lower average sperm production (James, 1980).

The publication of the meta-analysis by Carlsen *et al.*, prompted other researchers to assess data they had collected from various groups of men. In 1995, a study published by Auger *et al.*, compared data collected from 1351 fertile men volunteering for sperm donation in Paris between 1973 and 1992 (Auger *et al.*, 1995). This study reported an annual rate of decline of 2.1% in sperm concentration over this period (89 million/ml in 1973 to 60 million/ml in 1992) (Auger *et al.*, 1995). This study did not find any change in semen volume but did find a significant reduction in sperm motility and the number of normal spermatozoa (Auger *et al.*, 1995). When the data were re-analysed by year of birth a reduction in sperm concentration of 2.6% per later year of birth was observed (Auger *et al.*, 1995). A smaller study from Scotland which examined data from 577 men also found a relationship between sperm counts and year of birth (Irvine *et al.*, 1996). Donors born in 1959 had a median sperm concentration of 98 million/ml whereas those born after 1970 had a median sperm concentration in the region of 78 million/ml (Irvine *et al.*, 1996). A similar small study from Belgium analysed 416 consecutive candidate semen donors (Van Waeleghem *et al.*, 1996). This study recorded a decrease in mean sperm concentration from $71x10^6$/ml between 1977-80 to 58.610^6/ml between 1990-1995. However, the trend reported in these studies may not be indicative of a global phenomenon. Another study using data from the Toulouse region of France, found no change in sperm concentration between 1977 and 1992 (Bujan *et al.*, 1996) and similarly, a study from Finland reported no decrease in sperm concentration between 1958 and 1992 (Suominen and Vierula, 1993). It is worth noting that in the French studies, the reduction in sperm concentration was detected in the urban area whereas the rural area did not show any deterioration in sperm concentration. Currently, the largest retrospective review on semen quality contains data from 19,848 semen analyses from 7714 men undergoing IVF for tubal disease and having normal semen analysis prior to IVF (De Mouzon *et al.*, 1996). This study reported a significant decrease in semen quality with later year of birth, the mean sperm concentration for men born before 1939 being $92.5x10^6$/ml, falling to $77.1x10^6$/ml for men born after 1965 (De Mouzon *et al.*, 1996).

Studies from the USA have reported no deterioration in semen quality. A study by Fisch *et al.*, 1996 analysed data from three locations (Minnesota , n=600; New York, n=400; Los Angeles, n=221) collected prior to vasectomy (Fisch *et al.*, 1996). This study reported a small but significant increase in sperm concentration between 1970 ($77x\ 10^6$/ml) and 1994 ($89x\ 10^6$/ml) (Fisch *et al.*, 1996). The data has been criticized as some of the means are from several samples while other subjects are represented by one sample, also the analysis techniques varied between the different locations and over time. A study from Seattle compared data from 510 normal men using multiple semen samples collected between 1972-

1993 (Paulsen *et al.*, 1996). These men were a highly selective sample based on normal blood chemistry, endocrine profiles and physical examination. The authors calculated the mean sperm concentration from the 1972 cohort to be 46.5x 10^6/ml and in 1993 their regression analysis showed a weak statistically significant increase with time which the authors suggest is clinically insignificant (Paulsen *et al.*, 1996).

If semen quality is reducing, these studies suggest it may be a regional phenomena. This would support the hypothesis that factors in the local environment could have an impact on reproductive health. A study that compared the sperm concentration of the partners of women undergoing fertility treatment in London found a reduction in sperm concentration between the periods 1978-1983 and 1984-1989. However, the changes were only detected in men living in areas where their water supply was derived from the river Thames; no reductions were found in men living in other water supply areas (Ginsburg and Hardiman, 1992).

The changes observed in the above studies which have analysed sperm counts using later year of birth may support the reasoning that exposure to adverse conditions during *in utero* or during prenatal development may influence reproductive capacity in later life (Toppari *et al.*, 1996). The data however are inconclusive as all the studies have some flaws, in that they are all retrospective; data is collected from different subject populations using different selection criteria and recruitment methods. Also, semen analysis is performed using different methodologies between laboratories. Systematic analysis over time and within the same geographical locations are required to monitor this situation. Prospective studies on semen quality in fertile men are currently under way in the USA, Europe and Japan which are using standard procedures to try to avoid many of the criticisms of the current data. This study will hopefully determine region specific differences in semen quality and will serve as a reference point for future studies on semen quality.

1.2.2 Testicular Cancer

One of the strongest indicators that there is a problem with male reproductive health is the increasing incidence of testicular germ cell cancer. This has been rising steadily over the past few decades and in the UK it is now the commonest malignancy of men aged 15-45. Data from cancer registries show that a 2-4% per annum increase in testicular cancer rates is evident in the United Kingdom (Boyle *et al.*, 1987; Nethersell *et al.*, 1984; Pike *et al.*, 1987), the Nordic and Baltic countries (Adami *et al.*, 1996; Hakulinen *et al.*, 1986), Australia (Stone *et al.*, 1991), New Zealand (Pearce *et al.*, 1987; Wilkinson *et al.*, 1992) and the United States of America (Spitz *et al.*, 1986). There are marked geographical variations in cancer rate. For

example, Denmark has a 4 times higher rate of testicular cancer than Finland and in the USA it appears that Caucasians have a 3 times higher incidence of testicular cancer than African Americans (Spitz *et al.*, 1986). A study that re-evaluated the data from 9 registries around the Baltic sea by birth cohort rather than by year of diagnosis supports other data in showing that testicular cancer has increased in incidence with each later year of birth regardless of whether a country has a high or low incidence of testicular cancer (Ekbom and Akre, 1998). The aetiology of testicular cancer is not understood but it is believed that events occurring *in utero* during testis development leads to the persistence of fetal gonocytes within the testis leading to the formation of pre-invasive carcinoma in situ cells (CIS) (Skakkebaek *et al.*, 1987) (Ekbom and Akre, 1998; Rajpert-De Meyts and Skakkebaek, 1993; Savage and Lowe, 1990). It has been hypothesised that these cells can become invasive after increased exposure to hormones which generally occurs at puberty (Cortes *et al.*, 1987), (Rajpert-De Meyts *et al.*, 1998). Exposure of the developing fetus to androgens or oestrogens (Savage and Lowe, 1990) are known to be risk factors for testicular cancer (Toppari *et al.*, 1996). It is not known whether exposure to 'environmental oestrogens' is involved in the increasing incidence of testicular cancer which has been reported.

1.2.3 Congenital Abnormalities of the Male Reproductive Tract

1.2.3.1 Cryptorchidism

There are data from several reports suggesting that the incidence of cryptorchidism is increasing. Cryptorchidism is a condition in which the testicles do not descend fully into the scrotal sac but remain within in the abdomen or inguinal region. In some cases the testicles will automatically descend into the scrotum but other boys undergo orchidopexy (an operation to bring the testis into the scrotum). The data used to support an increase in cryptorchidism is not as strong as the testicular cancer data mainly because the studies use different diagnostic criteria to define a cryptorchid testis and some include various degrees of retractile testes. Also, the reported prevalence of cryptorchidism varies substantially between 0.03-13.4% using data from birth to one year of age from registers (Campbell *et al.*, 1987; Matlai and Beral, 1985; Mau and Schnakenburg, 1977; McIntosh *et al.*, 1954). In many studies racial and ethnic data are pooled and very few studies have examined the temporal changes in the incidence of cryptorchidism within the same geographical location and using identical criteria to define the condition. Data from England and Wales which examined the number of boys undergoing orchiopexy suggested an increase from 1.4% in a 1952 birth cohort to 2.9% in a 1977 birth cohort using the same diagnostic criteria (Chilvers *et al.*, 1984). Similarly, in Scotland the number of boys diagnosed with cryptorchidism showed a significant increase between 1961 and 1985 (Campbell *et al.*, 1987). A recent study has concluded that the

highest risk factor for both cryptorchidism and hypospadias is a low birthweight (Weidner *et al.*, 1999).

1.2.3.2 Hypospadias

Hypospadias is a congenital abnormality of the penis in which the urethral opening does not appear at the tip of the glans but can occur anywhere along the shaft of the penis. This is caused by incomplete closure of the urethral folds during penis development. There are a substantial number of reports in the literature which suggest that the incidence of hypospadias is increasing. However, as with cryptorchidism, the studies are more difficult to compare as the criteria used to define the condition and the inclusion/exclusion criteria are different amongst the published studies. The figures for the prevalence of this condition in the world literature varies considerably between 0.37 to 41 per 100,000 infants (Toppari *et al.*, 1996). Increases in hypospadias have been reported in the United Kingdom (Matlai and Beral, 1985), Hungary (Czeizel, 1985; Czeizel *et al.*, 1986), Sweden (Kallen *et al.*, 1986; Kallen and Winberg, 1982), Norway (Bjerkedal and Bakketeig, 1975) and Denmark (Kallen *et al.*, 1986).

In the United Kingdom the national register indicated the prevalence of hypospadias has risen from 7.3 per 10,000 births in 1964 to 16 per 10,000 in the 1980s. Data from 1990 indicated a reduction in incidence at 11.7 per 10,000 births (Toppari *et al.*, 1996). In Hungary the incidence of hypospadias was reported as 5.5 per 10,000 births in 1964 and increased to 23.9 per 10,000 births in 1978 and has since remained at this level (Toppari *et al.*, 1996). Results from similar studies examining the incidence of testicular cancer between in Caucasians and African Americans have concluded that the incidence of hypospadias is higher in Caucasians than African Americans (Toppari *et al.*, 1996).

The most recent report on the incidence of hypospadias and cryptorchidism examines the international trends using data obtained from countries participating in the International Clearinghouse for Birth Defects Monitoring Systems (ICBDMS) (Paulozzi, 1999). An upward trend in the rate of hypospadias in the USA was noted from 1970, increasing in incidence from 20 per 10,000 births to 36 per 10,000 births. Data from the Commonwealth group (Canada, Australia and New Zealand) showed values between 10-20 per 10,000 births in the early 1980s and similar values in the early 1990s; although there were fluctuations in the values there was little net change. Comparing data from Scandinavia (Denmark, Finland, Norway, Sweden) an upward trend was reported (except in Sweden) with rates more than doubling in Norway and Denmark between 1970-1993 (6 per 10,000 to 15 per 10,000 births). Finland reported a small increase in the milder forms of hypospadias. In the group

encompassing Northern Europe (England, France, Holland) and Japan, all the countries except Holland showed a net increase in hypospadias. The grouping which examined Ireland and the Mediterranean (Israel, Italy, Spain) only one region of Italy showed an increase in the incidence of hypospadias. Of the less affluent nations (classed as China, Czech Republic, Hungary, Mexico and South America) the rates recorded were relatively stable except Czechoslovakia which registered an increase (Paulozzi, 1999). Far fewer countries recorded data on cryptorchidism to the ICBDMS. In USA and Canada the national rates increased over 1970s-80s but this may have been due to more inclusive case definitions. In Norway and France there is no consistent trend detectable while in England the rates recorded dropped sharply in 1990 after the introduction of an "exclusion list" (Paulozzi, 1999).

The data collated by Paulozzi, (1999) does not indicate a general increase over time but increases were more frequently recorded within the more affluent nations. This data highlights the need for prospective, standardised studies to be performed in several geographical locations to monitor the true incidence of these conditions.

1.2.4 Changes in Wildlife

The changes that have been documented in the literature regarding declining male reproductive health are not confined to humans. The animal kingdom is also showing various degrees of abnormalities within the male reproductive system. The most well documented phenotypes include feminisation, reduced fertility, hatchability and viability of offspring and altered sexual behavior (Colborn and Clement, 1992). Although in most cases the causative agent/s are not known, researchers have linked these effects to endocrine disrupting chemicals. Many chemicals have been identified in the environment that can act as potential endocrine disrupters. However, exactly how these chemicals interact with each other to counteract or synergise endogenous pathways within a biological system and whether the levels of these chemicals are high enough to exert any biological changes is not known. It does appear, however, that more gross malformations are occurring in animals 'lower' down the phylogenetic tree with birds, reptiles, amphibians and fish showing the most pronounced effects.

In 1980, Lake Apopka, Florida, was badly polluted after a chemical spill with trichloroethane (DDT), and subsequently with its degradation metabolites DDD and DDE which are known to have oestrogenic, anti-androgenic, or endocrine disrupting effects (Bitman and Cecil, 1970; Bitman *et al.*, 1968). The lake suffered a decline in the number of juvenile alligators due to reproductive disorders induced by these chemicals (Guillette *et al.*, 1994). Male alligators had

abnormal germ cells within the testis and abnormally small phalli indicating that normal male sexual development had been permanently modified by exposure to high doses of the polluting chemicals.

Abnormal reproductive tracts have been found in many species of fish. Fish living in water downstream from kraft pulp mills (e.g. white sucker) showed signs of delayed maturation, smaller gonads, lack of secondary sex characteristics, reduced serum testosterone and a dysfunctional hypothalmic-pituitary-gonadal axis (Munkittrick *et al.*, 1991). The effects of polychlorinated biphenols (PCBs) have been investigated in Atlantic cod as dietary exposure to these compounds disrupts testicular spermatogenesis (Toppari *et al.*, 1996). In England, rainbow trout downstream of sewage treatment outflows display an increased incidence of hermaphroditism. These male fish produce large quantities of the egg protein vitellogenin which is normally only produced by female fish in response to oestrogen (Jobling and Sumpter, 1993; Purdom *et al.*, 1994). In a bioassay to assess vitellogenin production on exposure to xenoestrogens, a class of alkylphenol-polyethoxylates, a major group of surfactants found in sewage were shown to be oestrogenic to fish (Jobling *et al.*, 1996).

The Florida Panther, an endangered species of which there are only about 35 individuals, exhibits a wide array of reproductive abnormalities (Facemire *et al.*, 1995). The males have a low ejaculate volume, low sperm concentrations (>90% of sperm are morphologically abnormal) and exhibiting poor sperm motility. The incidence of cryptorchidism has increased since 1975; all but one of the nine cubs born since 1985 are cryptorchid. These abnormalities are thought to be due to the contamination of mothers with endocrine disrupting chemicals (Facemire *et al.*, 1995) but others have argued that these abnormalities could be a consequence of in-breeding.

In most cases there is no definitive proof that the changes observed in wildlife have occurred as a direct consequence of exposure to 'environmental oestrogens'. However, the changes reported in human reproductive health and the similarities reported in some animal species only serves to heighten awareness that there could be a problem. In species where the causative agent is known, the adverse effects on the reproductive tract have been induced only after exposure to high levels of the environmental contaminant. Therefore effects observed in wildlife must be interpreted with caution, and care must be taken not to extrapolate the causes directly with the changes observed in human reproductive health.

Many of the reported malformations described in both humans and wildlife are evident at birth and probably stem from changes induced during development. It may be possible that high levels of oestrogenic and/or anti-androgenic chemicals may interfere with the normal patterns of sexual differentiation and subsequent development of the gonads and accessory organs. It is therefore important to identify whether exposure to such chemicals, at doses comparable to those found in the environment, can act to interfere with normal sexual development. The current understanding of the sites of oestrogen and androgen action within the male reproductive tract is reviewed in section 1.6.

1.2.5 Diethylstilbestrol and Abnormalities in the Reproductive Tract of Males

The synthetic oestrogen, diethylstilbestrol (DES), was prescribed to pregnant women from the late 1940s to the early 1970s as a preventative measure to stop miscarriage and pregnancy complications (Palmund *et al.*, 1993). The results of a double-blind placebo-controlled study on the therapeutic value of DES demonstrated that it was not efficacious for the medical conditions for which it was being used (Dieckmann *et al.*, 1953). DES was associated with an increased rate of abortion, neonatal deaths and premature births (Brackbill and Berendes, 1978). The use of DES during pregnancy was banned in the early 1970s after it was discovered that a very rare form of vaginal cancer (clear cell adenocarcinoma of the vagina) had increased in incidence in young women whose mothers had taken DES (Herbst *et al.*, 1974). Millions of women across USA and Europe were exposed to DES and in addition it was used as an anabolic agent in livestock and was transferred into dairy products.

The sons of mothers exposed to DES during pregnancy also showed abnormalities in the structure and function of reproductive organs (Arai *et al.*, 1983; Stillman, 1982). These included an increased incidence of hypospadias, epididymal cysts, hypoplastic testes, cryptorchidism, microphallus and abnormal semen parameters (Gill *et al.*, 1977; Gill *et al.*, 1979; Henderson *et al.*, 1976). Several studies have recorded significantly lower sperm concentrations in DES exposed men (83 million per ml in DES exposed men as opposed to 123 million per ml in placebo exposed men) (Gill *et al.*, 1979) and other studies published similar findings (Stillman, 1982). However most DES exposed men are fertile and have sperm counts within the defined normal range (> 20 million per ml) (Wilcox *et al.*, 1995). There is however a significantly higher number of DES exposed men who have semen analysis scores which are rated 'severley pathological' (23% versus 5%) (Gill *et al.*, 1977). There is some evidence to suggest that DES induced a small increase in the incidence of testicular cancer (Vessey, 1989) which reached significance after meta-analysis (Toppari *et al.*, 1996). Men who were exposed to DES before 11 weeks of gestation (i.e. when the genital

tract is just forming) had double the risk of developing genital anomalies compared to men exposed to this chemical later in gestation (Wilcox *et al.*, 1995). This demonstrates the sensitivity of the fetus to exogenous chemicals during organogenesis and the importance of the timing of exposure.

1.2.6 Oestrogenic Chemicals Identified In The Environment

The industrialisation of the Western world over the last century has led to revolutions in manufacturing processes. Industry has developed thousands of compounds, produced new materials and products that have become part of everyday life. Industrial manufacturers are capable of producing thousands of tonnes of chemicals every year. Some of these products are involved in the production of pesticides, plastics, detergents, petrol and many other household commodities. When these compounds were initially manufactured they were not considered to have potentially harmful effects on man. Therefore the release of tonnes of such chemicals into the environment every year was of no real concern. The major change in this thinking probably occurred during the 1960s when it became clear that some organochlorine pesticides were having detrimental effects on wildlife. Thirty years later, concern has been raised again due to the possibility that synthetic chemicals in the environment may be having an adverse effect on human reproductive health.

An increasing number of synthetic chemicals have been identified as hormonal mimics either *in vitro* or *in vivo*. Currently, the major classes of these compounds include some members of the following groups: organochlorine pesticides, polychlorinated biphenols, alkyl- and bis-phenolic compounds and phthalate esters. However, only a small fraction of chemicals within the environment have been tested and it is not possible to predict accurately whether a compound is oestrogenic or not from its chemical structure, therefore this list will probably continue to increase for the foreseeable future.

Organochlorine pesticides include methoxychlor, kepone (chlorodecane), dieldrin and 1,1,1-trichloro-2,2-bis (*p*-chlorophenyl) ethane, better known as DDT. The use of DDT was banned in developed countries in the 1960s but it is still widely used in developing countries. However, DDT is a persistent chemical in the environment with a half-life of > 60 years. It is also lipophilic and accumulates in body fat. There are several isomers of DDT, of which *o,p'*-DDT has the most potent oestrogenic activity as determined by both oestrogen receptor binding (Kelce *et al.*, 1995) and uterotrophic activity *in vivo* (Gellert *et al.*, 1972). However, the major metabolite of DDT found in the body is *p,p'*DDE which has potent anti-androgen effects both *in vitro* and *in vivo* (Kelce *et al.*, 1995). Other compounds such as methoxychlor

and chlordecone are also oestrogenic and administration of either compound to neonatal female rats induces accelerated vaginal opening and infertility (due to persistent oestrus in adulthood) (Colborn and Clement, 1992).

Industrial chemicals such as polychlorinated-biphenols (PCBs) are also widespread and persistent in the environment. There are over 200 congeners of PCBs, some of which possess oestrogenic activity while some others have anti-oestrogenic activity (Turner and Sharpe, 1997). Another group of industrial chemicals, the alkylphenol polyethoxylates (APEOs) are widely used as surfactants in many household products such as plastics, detergents, petrol additives, paints, cosmetics and condom spermicides. Up to 60% of these chemicals enter the aquatic environment. During sewage treatment, microbes break down APEOs, and degradation products are produced, some of which have oestrogenic activity (octylphenol and nonylphenol) (Turner and Sharpe, 1997). Similarly, bisphenol A, another component of polycarbonate plastics, acrylic resins, xeroxing chemicals and the lacquer coating of food cans has been shown to be oestrogenic. Finally, another group of chemicals, the phthalates which are used as plasticizers in the production of plastics and resins are among some of the most ubiquitous synthetic environmental chemicals. The majority of phthalate esters are not oestrogenic but butyl benzyl phthalate and di-n-butyl phthalate are oestrogenic *in vitro* (Turner and Sharpe, 1997).

All the synthetic environmental oestrogens described to date are weak oestrogens. They are at least 1000 times less potent than oestradiol (Arnold *et al.*, 1996). This suggests biological effects would only occur after exposure to very high levels. But this situation is complicated by the fact that these chemicals may be capable of causing additive, synergistic or antagonistic effects. However, a recent study examined the sons of farmers and gardeners for genital tract abnormalities, since some of the heavily used chemicals within these occupations have been shown to be oestrogenic and possess other hormone-disrupting effects (Weidner *et al.*, 1998). The results indicated a significant increase in the risk of cryptorchidism but not hypospadias in the sons of mothers (but not fathers) working in these occupations (Weidner *et al.*, 1998). This suggests that occupational exposure to some of these chemicals may be high enough to induce an increase in congenital abnormalities.

There is another source of oestrogens which occur naturally in the environment, termed phytoestrogens (plant oestrogens) that are likely to be a more significant source of human oestrogen exposure than synthetic oestrogens. Phytoestrogens are consumed largely via the diet and are present in substantial levels in soya, grains, vegetables, and fruits. Phytoestrogens

can be grouped into 3 main subsets: isoflavones (e.g. genistein, daidzein), coumestans/lignans (e.g. coumesterol) and mycoestrogens (e.g. zearanalone) (Kaldas and Hughes, 1989). Unlike many of the synthetic compounds, phytoestrogens do not bioaccumulate in body fat and are readily metabolised. This is one source of oestrogen which has undoubtedly increased in the developed world in recent years particularly through the addition of soy-based proteins to many convenience foods. There are contradictory views regarding the potential benefits and risks of increasing our consumption of phytoestrogens. One argument suggests that a high phytoestrogen diet, as is prevalent in Japan, may give some protection against breast, colon and prostate cancer (Messina *et al.*, 1994). However, a high dietary intake of phytoestrogens has been shown to cause reproductive abnormalities in sheep (Kaldas and Hughes, 1989) and can prolong the follicular phase of the menstrual cycle in humans (Cassidy *et al.*, 1994). Due to the increasing use of phytoestrogens in the Western diet (and also in baby foods and formula feeds) it is important to determine whether an increased dietary consumption of these chemicals has any effect (positive or negative) on reproductive health.

1.3 Anatomy of the Male Reproductive System

The male reproductive tract consists of two testes, each of which is attached to a highly coiled epididymis that ends in a straight vas deferens (see Figure 1.1). The vas deferens loops around the bladder and merges with the ducts of the seminal vesicles and then enters the prostate gland and joins the urethra which extends along the length of the penis (Curtis and Barnes, 1989).

1.3.1 The Testis

The testis is an ovoid structure encapsulating the seminiferous tubules which contain the developing germ cells. The testicular capsule (tunica albuginea) is a fibrous layer which covers the testis (Setchell *et al.*, 1994). It is composed of several layers of fibroblasts, collagen and smooth muscle cells. These muscle cells are capable of rhythmic contractions and may aid sperm transport out of the testis. The testicular capsule is also regarded as important for maintaining interstitial pressure within the testis and is important in controlling blood flow into the testis (Setchell *et al.*, 1994).

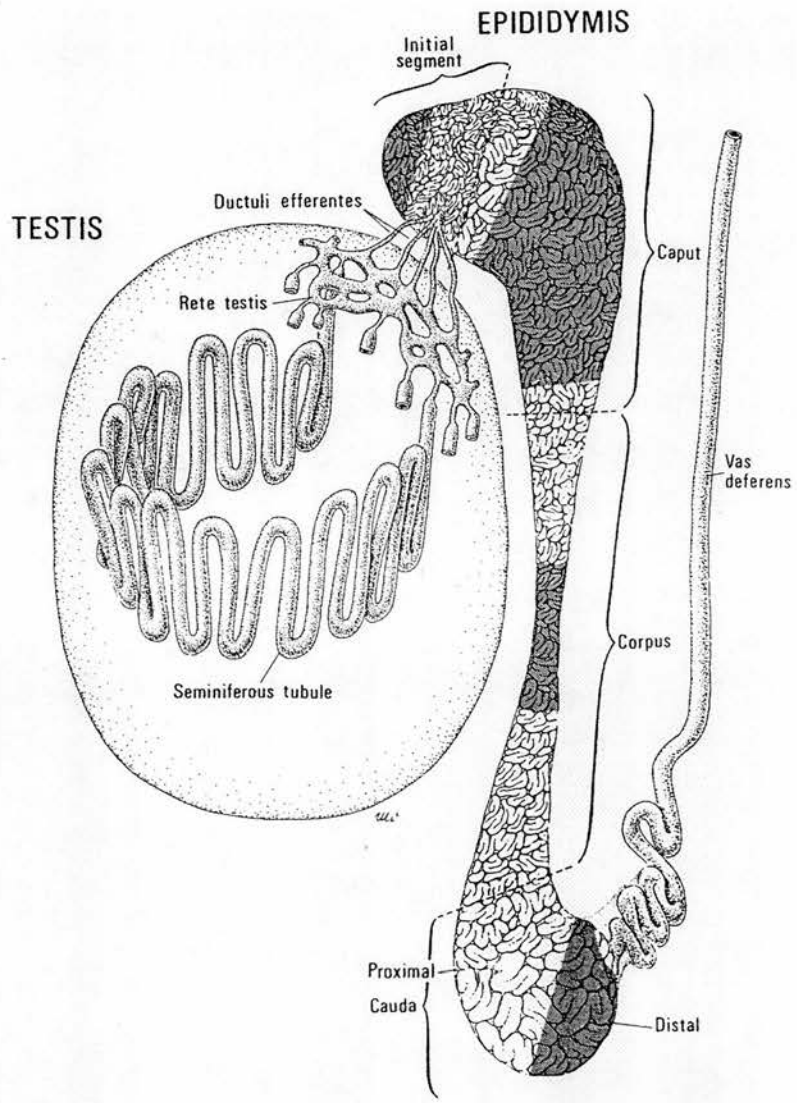

Figure 1.1 Diagram of the Testis and Excurrent Duct System

Illustration of the anatomy of the testis, rete testis, efferent ducts, the regions of the epididymis and the vas deferens and their proximity to each other. Adapted from (Robaire and Hermo, 1988).

The majority of adult testicular volume comprises seminiferous tubules which consist mostly of germ cells and the only somatic component, the Sertoli cells. The seminiferous tubules are coiled loops which have both ends attached to the rete testis. In the rat there are approximately 30 tubules per testis (Setchell *et al.*, 1994). A layer of peritubular myoid cells surrounds the basement membrane of each seminiferous tubule and these cells are also able to contract,

which may aid sperm transport. The regions between seminiferous tubules contain interstitial cells, nerves, blood and lymph vessels. The interstitial cells comprise Leydig cells (hormone secreting cells), as well as immune cells (mast cells and macrophages).

1.3.2 Spermatogenesis

In order to understand how sperm counts could be affected by exogenous chemicals it is important to have a basic understanding of how the process of spermatogenesis is constructed. It is only by understanding how sperm are produced and what processes determine the efficiency of spermatogenesis that insight can be gained into the processes that could be vulnerable to disruption by oestrogenic chemicals.

1.3.2.1 The Organisation of Spermatogenesis

Spermatogenesis is a complex process which transforms a round germ cell into a flagellated spermatozoon. There are morphologically and spatially defined stages that each developing germ cell passes through and these comprise the spermatogenic cycle.

The developing germ cells are enclosed within the seminiferous tubules of the testis. At the base of the tubules lie the stem cells (spermatogonia; termed A_0 in the rat and A_d in humans) and the Sertoli cells. A schematic diagram of a cross section through a Sertoli cell is shown in Figure 1.2. The cytoplasm of the Sertoli cell extends in towards the lumen of the seminiferous tubule. These cells are essential to spermatogenesis as their extended cytoplasm engulfs the developing germ cells and supplies them with nutrients and hormones.

In Figure 1.2, immature germ cells (spermatogonia) can be observed at the base of the seminiferous tubule and as they differentiate the germ cells move towards the lumen where they are released upon completion of spermatogenesis. At any one time, the Sertoli cell has to support several different germ cell types at different stages of development. Due to the defined time required for the maturation of one germ cell type into another there are patterns of germ cell complements which are always viewed in cross-section together. These germ cell complements are called stages. There are XIV stages identified in rat spermatogenesis and these are collectively referred to as the spermatogenic cycle (Sharpe, 1994). As each stage is composed of germ cells at various developmental stages, a single germ cell passes through the spermatogenic cycle 4.5 times. A single passage through the cycle takes ~12.5 days and the duration of rat spermatogenesis is between 51 and 53 days (Sharpe, 1994).

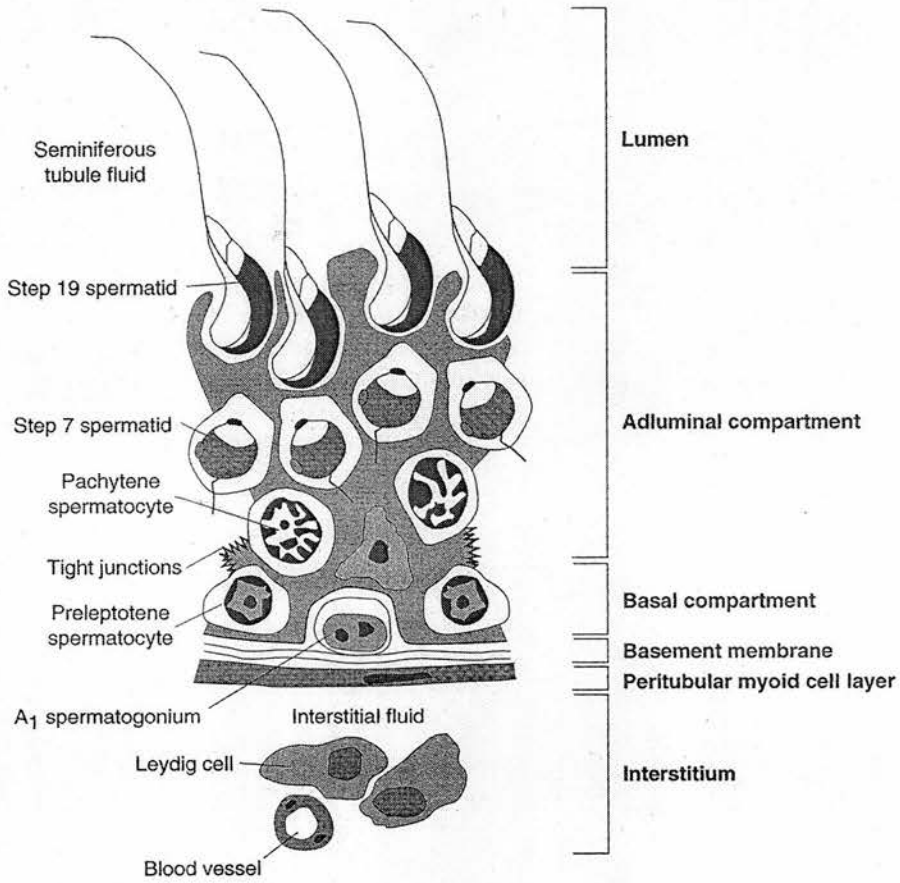

Figure 1.2 Diagram of a Cross section through a Sertoli Cell

Illustrates both the anatomical arrangement of the seminiferous tubule and the close association between the Sertoli cell of the Seminiferous tubule and the developing germ cells. Adapted from (Sharpe, 1994).

At the initiation of a spermatogenic cycle the spermatogonia divide, one of the daughter cells repopulates the stem cell population while the other undergoes a series of mitotic divisions. In rat spermatogenesis there are six mitotic divisions and the cells pass successively through A_0, A_1, A_2, A_3, A_4 and an intermediate stage to become B spermatogonia. The daughter cells of the final mitotic division enter meiosis and are termed preleptotene spermatocytes. These cells pass through the Sertoli cell tight junctions and enter the adluminal compartment. They are no longer able to gain independent access to nutrients and hormones derived from either the blood stream or interstitial fluid and become dependent on the Sertoli cell to support them throughout spermatogenesis (Sharpe, 1994).

The process of meiosis involves DNA synthesis (preleptotene spermatocytes), RNA synthesis (pachytene spermatocytes) and, upon completion of meiosis, a reduction division to produce 4 haploid round spermatids. The differentiation of the haploid cells into spermatozoa occurs during the process of spermiogenesis. Spermiogenesis has been separated into 19 morphologically defined steps. During this process the nucleus compacts its DNA which thus becomes transcriptionally inactive. The nucleus becomes overlain with an enzyme filled sack called the acrosome which, at fertilization, aids sperm penetration of the vestiments surrounding the oocyte. The developing spermatids lose most of their cytoplasm and organelles during spermiogenesis and develop a flagellum which is rich in mitochondria and will allow the cell to be motile after ejaculation (Sharpe, 1994).

1.3.2.2 What Determines Sperm Numbers

There are a few factors which are important in determining the overall sperm count. For examples, the number of Sertoli cells, the number of germ cell mitoses and rate of degeneration; these will be discussed briefly below.

1.3.2.2.1 The Relationship Between Sertoli Cells and Sperm Production

The number of Sertoli cells within the testis is essential in determining both testicular size and daily sperm production in the adult animal (Russell and Peterson, 1984). As each Sertoli cell can only support a defined number of germ cells through spermatogenesis at one time, the number of Sertoli cells determines the maximum achievable sperm production for each testis. The germ cells comprise most of the testicular volume and the quantity of germ cells is limited by the number of Sertoli cells, therefore, indirectly, Sertoli cells determine adult testis size (Sharpe, 1998). So, what determines the number of Sertoli cells?

The number of Sertoli cells is determined prior to the onset of spermatogenesis. Sertoli cells divide when they are immature/undifferentiated in fetal/neonatal life and, in some species, in early puberty (Sharpe, 1994). In the rat, Sertoli cell multiplication occurs from embryonic day 19 through until day 15 postnatal (Orth, 1984; Orth, 1982) while in humans, Sertoli cell proliferation occurs in neonatal and probably in the pre-pubertal period but not thereafter (Cortes *et al.*, 1987). The most important factor which can increase the rate and duration of Sertoli cell division is the gonadotrophic hormone FSH (Orth, 1982). The neonatal suppression of FSH in rats (i.e. during the period of Sertoli cell replication) leads to a permanent reduction in Sertoli cell numbers (Sharpe, 1994), (Sharpe *et al.*, 1998a). Oestrogen is a powerful suppressor of FSH secretion in males, particularly neonatally (Arai *et al.*, 1983), (Sharpe *et al.*, 1998a) and neonatal administration of high doses of the potent synthetic

oestrogen DES can permanently lower Sertoli cell numbers though this may result, in part, from direct effects of DES on the Sertoli cells (Sharpe *et al.*, 1998a). In contrast, the administration of moderate levels of oestradiol to neonatal rats does not induce an alteration in Sertoli cell numbers (Cook *et al.*, 1998). These studies suggest that oestrogens/oestrogenic compounds can inhibit Sertoli cell replication but possibly only at high concentrations. This suggests that oestrogenic chemicals could potentially influence the multiplication of Sertoli cells, however since these chemicals are weak oestrogens it seems unlikely that humans would consume enough of these compounds to induce a biological effect.

Whether or not xenoestrogens in the environment or other environmental/lifestyle changes could cause a reduced sperm output by this mechanism is not known but this is the basic tenet of the hypothesis put forward in 1993 to suggest a mechanism behind the changes reported in the literature (Sharpe and Skakkebaek, 1993). Using the rat as a model, various xenoestrogens were administered during the period of Sertoli cell replication and induced small (8-12%) but significant reductions in testis size and daily sperm production in adult rats (Sharpe *et al.*, 1995), although these findings have proved difficult to repeat (Sharpe *et al.*, 1998b).

1.3.2.2.2 The Efficiency of Spermatogenesis

Another factor which could influence the sperm output of an animal is the efficiency of spermatogenesis. This varies greatly between species. For example, the average number of elongate spermatids per Sertoli cell is 10.3 ±1.6 in rats in comparison with only 3.9±0.5 for human Sertoli cells (Sharpe, 1994). Similarly, when the daily sperm production (DSP) per gram of testis is compared, humans produce only 4.4 $x10^6$/g whereas the rat is capable of producing 24 $x10^6$/g. Humans have the most inefficient spermatogenesis of any mammal so far described and this is often linked to the manner in which the spermatogenic cycle is arranged within the seminiferous tubule. Rats have a segmental arrangement along the seminiferous tubule which means that all of the Sertoli cells in a cross section have germ cell complements at the same spermatogenic stage. Humans however, have a helical arrangement of stages which means that Sertoli cells in a single cross section may be in contact with germ cells at several different stages of the spermatogenic cycle which may lead to a suboptimal microenvironment for the developing germ cells (Sharpe, 1994). Why this helical arrangement evolved is unknown. The lower DSP/g may also stem from the number of mitotic divisions. In humans there may be as few as 3 divisions compared to 6 in the rat (see Figure 1.3) which would halve the number of germ cells entering meiosis in comparison to the rat.

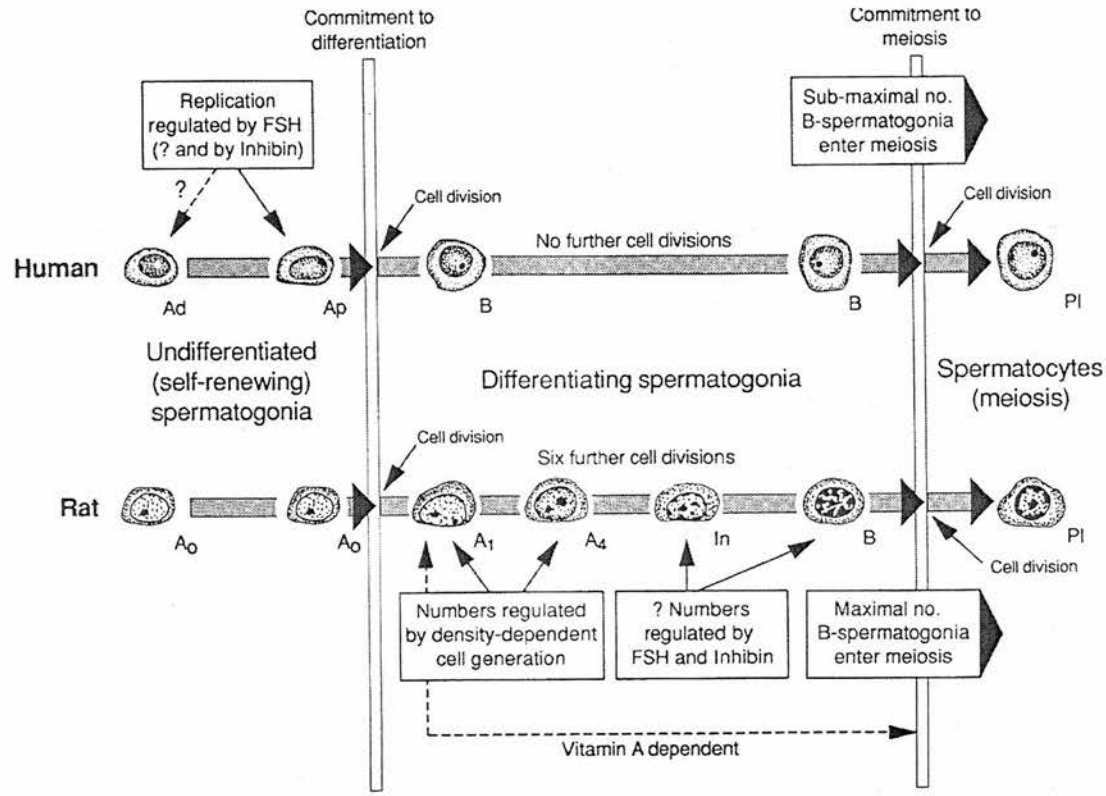

Figure 1.3 Illustration of the Differences between Rat and Human Spermatogenesis
These differences may account for the inefficiency of human spermatogenesis when compared to the rat. Adapted from (Sharpe, 1994).

Each preleptotene spermatocyte which enters meiosis should give rise to four haploid round spermatids but this ratio is never attained as meiosis is a major point in the spermatogenic cycle at which germ cell degeneration occurs and this happens at an increased frequency in humans (Sharpe, 1994). The most common stage for germ cell degeneration to occur in all mammals is during Metaphase II of meiosis. In rats there is a 4-6% shortfall in the expected number of spermatids after meiosis but in humans, this value is closer to 40% and appears to rise with age (Johnson, 1986) (Johnson *et al.*, 1990). Faulty meiotic divisions have been suggested as one cause which may account both for the high level of germ cell loss and for the high numbers of abnormal spermatozoa found within the normal human ejaculate (Sharpe, 1994), (Skakkebaek *et al.*, 1973). Germ cell degeneration can also occur during the mitotic divisions of spermatogonia and during nuclear condensation of spermatids, however loss at these stages is minor by comparison to losses at meiosis (Sharpe, 1994). Alterations in the level of FSH may also be important in determining the number of spermatogonia entering meiosis (see Figure 3). In primates, including humans, FSH is a factor involved in stimulating

spermatogonial cell division. The administration of FSH to adult cynomologous monkeys (Weinbauer and Nieschlag, 1991) increases the number of B spermatogonia, spermatocytes and spermatids over the treatment period. This effect was induced by an FSH induced increase in Ap spermatogonia (Sharpe, 1994). These data suggest that the Sertoli cell is not at maximum capacity, thus, theoretically the administration of FSH could improve the efficiency of spermatogenesis and a reduction could cause it to fall. However, there is little evidence that suppression of FSH in normal men causes any major decline in sperm counts if adequate testosterone levels are maintained (Bremner *et al.*, 1981).

From the above description, human spermatogenesis is very inefficient in comparison to most mammals. There appear to be several stages in the spermatogenic process which are susceptible to germ cell degeneration. In the adult testis the germ cells are responsible for most of the testicular volume so a large loss in germ cells would be expected to be associated with a reduction in testis weight. Whether exposure to oestrogenic chemicals could increase the rate of germ cell degeneration or decrease the rate of mitotic divisions and hence the number of B spermatogonia is unknown but exposure to DES and some environmental compounds has been demonstrated to decrease testicular weight and DSP in rats after gestational/neonatal exposure (Sharpe *et al.*, 1995). This decline was presumed to occur due to a reduction in FSH which induced a reduction in Sertoli cell numbers, however no studies have been performed to exclude germ cell degeneration or decreasing numbers of B spermatogonia as possible mechanisms for these decreases.

1.3.3 The Rete Testis

The terminal regions of the seminiferous tubules which open into the rete testis are termed transitional zones or tubuli recti (see Figure 1.4). This region of the epithelium contains no germ cells and is lined by cells resembling Sertoli cells (Roosen-Runge, 1961). The distal end of this region is an extension of the rete proper and in the human up to six seminiferous tubules can join with a single tubulus rectus (Roosen-Runge and Holstein, 1978).

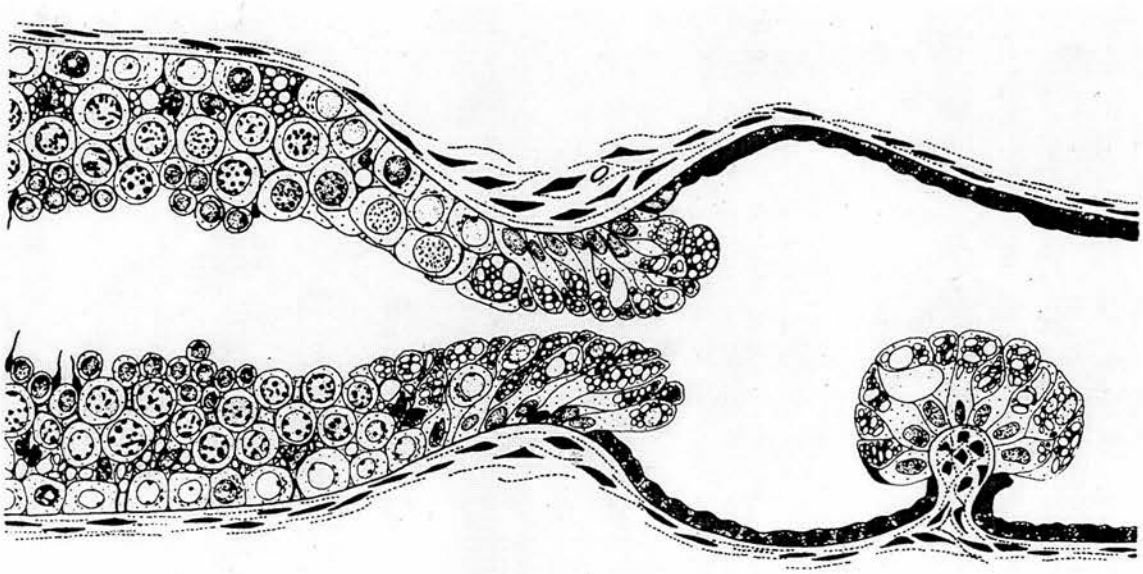

Figure 1.4 Diagram of a Testis-Rete Testis Junction

Illustrating the terminal segment (tubulus rectus) of a seminiferous tubule and the sharp change in morphology to the rete testis. Adapted from (Setchell *et al.*, 1994).

Under light microscopy the terminal segment has no lumen unless the testicular pressure is raised by efferent duct ligation (Osman and Ploen, 1978). Normally the tall columnar cells occlude the lumen due to their long cytoplasmic processes which some investigators have described as 'plug-like' structures and suggest these function as a valve to prevent fluid reflux back into the seminiferous tubule (Perey *et al.*, 1961; Roosen-Runge, 1961). Another function of the long cytoplasmic processes of these 'Sertoli cells' could be either resorption or secretion (Waites and Setchell, 1990).

The rete testis is often described as a labyrinth-like structure due to its network of interconnecting channels. The epithelium lining the rete changes from columnar in early postnatal life to cuboidal/squamous in the adult rete testis (Fisher *et al.*, 1997), (Dym, 1976). The location of the rete testis differs between species. In primates and rats the rete is situated under the tunica albuginea where it connects with the efferent ducts of the epididymis (Roosen-Runge, 1961) whereas in sheep the rete forms a cavity in the centre of the testis. Based on its anatomical position this structure can be described as having three parts, intratesticular, intratunical and extratesticular (Roosen-Runge, 1961). Of these regions, the intratesticular region is the largest.

The ultrastructural characteristics of the adult rat rete testis epithelium have been examined by electron microscopy (Leeson, 1962). The epithelium lining the central cavity of the rete appears squamous whereas the regions connecting with the tubuli recti and efferent ducts are cuboidal (Leeson, 1962). The cuboidal cell contains a multilobed nucleus which is situated towards the basement membrane rather than the lumen. The epithelial cell membranes of the rete testis have many specialised features, adjoining lateral membranes show complex interdigitating connections which are often associated with desmosomes. The role of these complex junctions is not known. The basal membrane of the epithelial cells is smooth and parallel to the basement membrane. The apical membrane shows numerous slender microvilli, pseudopodial projections associated with cytoplasmic vesicles and occasional cilia (Leeson, 1962). Whether these cilia are functional is doubtful (as no centrioles have been identified) but they may function as chemoreceptors (Leeson, 1962). The presence of microvilli, cytoplasmic vesicles and numerous mitochondria indicates that the epithelium is metabolically active and probably involved in fluid transport (Leeson, 1962). To support this, Morales and colleagues observed numerous small invaginations on apical, basal and lateral cell membranes corresponding to either the formation or fusion of pinocytotic vesicles (Morales *et al.*, 1984). The rete testis is embedded in highly vascular, loose fibro-connective tissue that lies close to the testicular artery and is covered by the mediastinal venous plexus (Free and Jaffe, 1972). The connective tissue of the rat rete testis consists of elongate fibroblasts. A "looseness" becomes increasingly evident around days 22-27 postnatal which probably reflects an increase in hydration (Nykanen, 1980). The rete testis epithelium is firmly attached to the basement membrane which may be to prevent compression and or obliteration of the lumen during changes in testicular pressure (Leeson, 1962).

1.3.4 The Efferent Ducts

The efferent ducts are a series of ductules connecting the rete testis to the epididymis. The number of efferent ducts varies between species; only five or six have been reported in humans (Jonte and Holstein, 1987; Saitoh *et al.*, 1990) and between two and nine have been isolated in the rat (Cooper and Jackson, 1972) (Dym, 1976) (Gutroff *et al.*, 1992). The efferent ducts become increasingly coiled as they near the epididymis forming the bulbous coni vasculosi which connects to the initial segment of the epididymis (Setchell *et al.*, 1994). The efferent ducts of the rat converge into a single tubule which continues as the initial segment of the epididymis (MacMillan, 1953) though some investigators believe that two separate branches of efferent ducts join with the epididymis (Cooper and Jackson, 1972). The lining of the efferent duct epithelium is largely composed of two cell types, ciliated and nonciliated cells, but some investigators have also reported intraepithelial lymphocytes and

basal cells (Ramos and Dym, 1977; Setchell *et al.*, 1994). These cell types have a low capacity for protein synthesis but have a well developed endocytotic apparatus that is active in fluid resorption (Ramos and Dym, 1977). Beneath the epithelium is a basal lamina which lies adjacent to a layer of periductular connective tissue which contains fibrocytes, abundant collagen fibres and a layer of smooth muscle cells (Ramos and Dym, 1977).

1.3.4.1 Ciliated Cells

The apical surface of the cell has many cilia and a few microvilli. The cilia contain the typical 9 + 2 arrangement of microtubules and terminate in a basal body. The well developed ciliary roots frequently reach to the perinuclear zone far beyond the apical cytoplasm (Ramos and Dym, 1977). These cells have large ovoid or spherical nuclei which are located in the middle or apical regions of the cells (Ramos and Dym, 1977). The nuclei contain electron lucent euchromatin with prominent nucleoli (Ramos and Dym, 1977). The cell cytoplasm contains numerous round or short rod-shaped mitochondria which are most concentrated around the supra- and perinuclear areas of the cells (Ramos and Dym, 1977). The cytoplasm contains numerous microfilaments, free ribosomes and polyribosomes; however, neither the rough nor smooth endoplasmic recticulum are well developed (Ramos and Dym, 1977). There is some rough endoplasmic recticulum and Golgi apparatus in the supranuclear and apical regions of the cells (Ramos and Dym, 1977). The function of the cilia is not clear. Initially they were thought to propel the sperm through the duct but the cilia on opposing sides of the epithelium beat in opposite directions therefore they probably induce a constant current in the fluid causing it to mix (Ilio and Hess, 1994).

1.3.4.2 The Nonciliated Cells (Apical or Principal Cells)

The surface of the apical cell is covered in microvilli of a similar height. Canaliculi extend down into the cytoplasm between the microvilli and in some cells appear continuous at their ends with coated vesicles called tubular coated pits (Hermo *et al.*, 1988; Ramos and Dym, 1977). The nonciliated cells have a dense nucleus which contains heterochromatin and is situated near the basal lamina (Ramos and Dym, 1977). The nucleus is smaller and has numerous deep infoldings of the nuclear envelope compared to the ciliated cell. The cell cytoplasm contains fewer mitochondria than the ciliated cell but similarly the endoplasmic recticulum is not well developed (Ramos and Dym, 1977). The most notable entities within the cytoplasm of the nonciliated cell are the many micropinocytotic vesicles, membrane bound dense bodies and numerous vacuoles it contains (Ramos and Dym, 1977). These characteristics provide strong evidence for this cell having a role in endocytosis.

1.3.4.3 Nonciliated Cells and Endocytosis

Even the early light microscopic studies into the function of the efferent ducts suggested a role in endocytosis (Mason and Shaver, 1952; Van Wagenen, 1925). This suggestion was supported by electron microscopy studies which employed the use of electron dense tracers to examine the fate of substances within the lumens of the efferent ducts (Goyal and Hrudka, 1980; Hermo *et al.*, 1988; Ladman, 1967; Yokoyama and Chang, 1971). Changes in the composition of luminal fluid between the start of the efferent ducts and the beginning of the initial segment of the epididymis have been detected using micropuncture techniques (Levine and Marsh, 1971) (Jenkins *et al.*, 1980; Turner, 1984), providing further evidence that there is a dynamic flux of fluids, proteins and ions across the efferent duct epithelium.

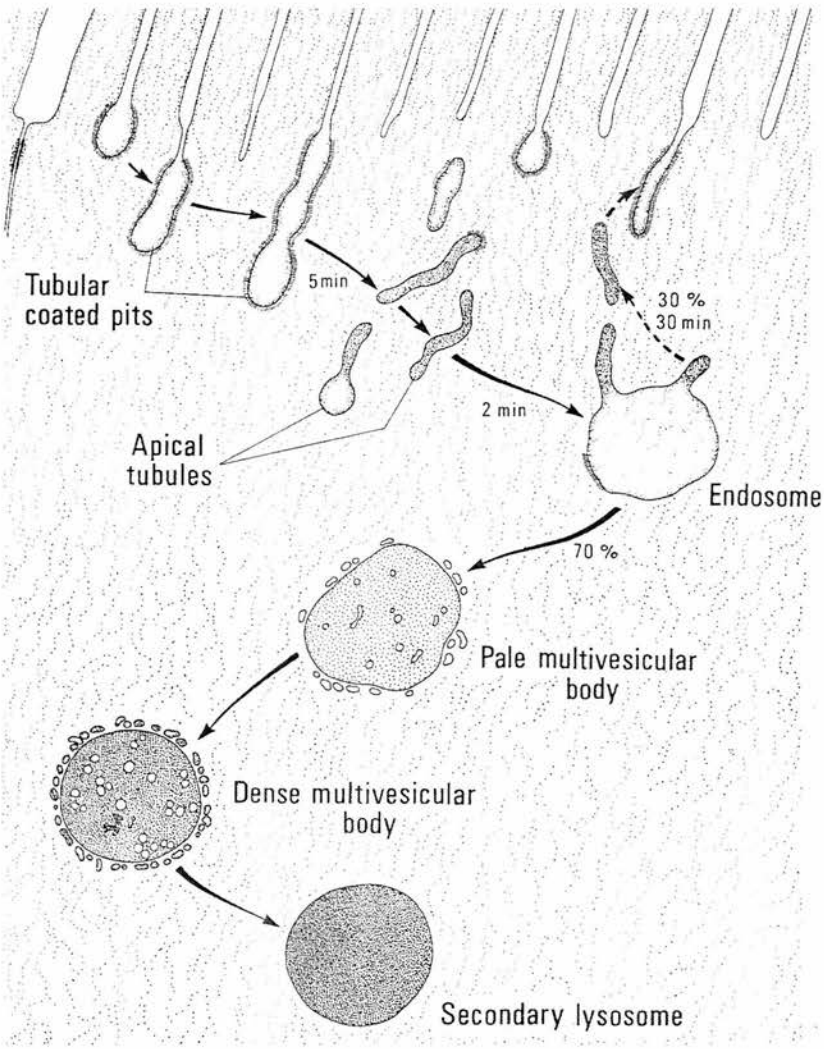

Figure 1.5 Demonstrating Nonciliated Cell Endocytosis

Illustrating the process of endocytosis within the nonciliated cell of the efferent duct epithelium as determined after the injection of electron dense tracers. Adapted from (Hermo *et al.*, 1988).

The apical region of the nonciliated cell cytoplasm contains many specialised vesicles and coated tubules which are important in endocytosis (see Figure 1.5). Two types of endocytosis have been described, adsorptive and fluid phase, depending whether or not the molecule binds to the apical plasma membrane. If the molecule does bind via a specific or non-specific receptor and is then internalised by pinocytosis this is classed as adsorptive endocytosis. When a molecule is suspended in fluid and does not bind to the surface membrane, but is still internalised via pinocytotic vesicles, this process is termed fluid phase (or bulk) endocytosis (Morales *et al.*, 1984). Experimentally, tracers such as cationic ferritin (CF) which bind electrostatically to anionic groups at the cell surface have been used to examine adsorptive endocytosis, and horseradish peroxidase (HRP) is extensively used as a marker to demonstrate fluid phase endocytosis The pathway through the cell is identical whether the substance is internalised by adsorptive or fluid phase endocytosis and is shown diagrammatically in Figure 5. Tubular coated pits (TCP) are connected to the apical plasma membrane. Beneath these are apical tubules (AT) which show a partial coat (Hermo *et al.*, 1988). Using CF both TCP and AT were shown to exhibit similar patterns of labeling. The amount of tracer found within both these structures peaked after 25 and 60 minutes suggesting that the tracer was being recycled within the cell as only one injection of tracer was given. TCP were labeled after 30 seconds, before any tracer was identified in AT, indicating that tracer enters the TCPs first (Hermo *et al.*, 1988). Molecules enter sequentially, TCP, AT, endosomes, multivesicular bodies and secondary lysosomes. It is thought that AT bud off from TCP and a portion (30%) of these fuse to form endosomes and then recycle back to the cell surface while the other 70% transform into endosomes, multivesicular bodies and secondary lysosomes (See Figure 5) (Hermo *et al.*, 1988). AT have been found in other epithelia involved in endocytosis and membrane recycling, e.g. the proximal convoluted tubule of the kidney (Graham and Karnovsky, 1966).

1.3.5 The Epididymis and Vas Deferens

The epididymis is a single highly convoluted duct that extends from the anterior to the posterior pole of the testis and is held closely to the testicular capsule by connective tissue. The epididymis is grossly divided into 3 regions, the caput, corpus and cauda (head, body and tail respectively), but other investigators have made more detailed divisions based on morphological and histological observations which have separated the epididymis into between 6 and 12 regions. There are many different cell types within the epididymis some of which are region specific and the roles of which are not clearly understood. The major cell type within the epididymal epithelium is the principal cell, which has stereocilia on its apical brush border. This cell type is found throughout the epididymal duct. The other cell types

include basal cells, clear cells, apical cells and halo cells. Functionally the caput region of the epididymis is important for maturational changes to the spermatozoa and the distal regions are thought to be important for storing mature sperm in a quiescent state. At the distal region of this duct the epididymis straightens out and continues as the vas deferens.

The initial segment of the epididymis continues from the efferent ducts. The principal cells in this region are taller than in other epididymal regions with tall microvilli which form a brush border. Only the initial segment contains narrow cells, so called as they have an ill-defined narrow base contacting the basement membrane, but these cells give rise to clear cells which are found in the rest of the epididymis. Both the columnar and narrow cells derive from a common undifferentiated stem cell (Clermont and Flannery, 1970). Principal cells and basal cells are derived from a columnar cell and the specific cell types are evident after postnatal day 28 (Sun and Flickinger, 1979). The principal cells found in the remainder of the epididymis are shorter but have a larger apical surface in contact with the lumen and with the basement membrane via foot-like processes (Robaire and Hermo, 1988). These cells show specialised adaptations for endocytosis with coated pits comprising a clathrin-like lattice coat within the apical membrane. There are also numerous large uncoated vesicles and apical tubules. The lateral plasma membranes are smooth and show no interdigitations with neighbouring cells.

Clear cells have only been identified in the rodent epididymis and have a higher endocytotic activity than principal cells (Robaire and Hermo, 1988). Basal cells have been identified in all species studied. These cells can be small, round or elongated in appearance with a large nucleus and little cytoplasm. They are often situated between two principal cells and have a large area of contact with the basement membrane. Basal cells have few cytoplasmic features suggesting they are not very metabolically active and their role is unknown. Throughout the epididymal epithelium there are halo cells, often referred to as lymphocytes (Robaire and Hermo, 1988). These cells migrate into the epididymis during postnatal development. Similar cells are also found surrounding seminiferous tubules and share many features with monocytes from peripheral blood.

The functions of the epididymis are generally cited as the transit of spermatozoa to the vas deferens, the acquisition of sperm motility, sperm maturation and storage in the cauda epididymis. Transit time through the epididymis in mammals is approximately 10 days. Sperm transit is aided by neuronal input which induces contraction of the periductular myoid cells (Robaire and Hermo, 1988). The length of time that sperm are stored in the cauda epididymis is variable and depends on ejaculatory frequency (Robaire and Hermo, 1988).

Sperm also acquire the ability to fertilize during transit through the epididymis but it is unclear how much sperm maturation is driven by the epididymal epithelium and how much maturation is a sperm driven process.

The epididymis is regarded as being androgen dependent through the results of numerous castration and testosterone replacement studies (Brooks, 1979; Karkun *et al.*, 1974; Robaire *et al.*, 1985). It has been shown to atrophy when the testis is removed. Testosterone treatment only partially maintains epididymal weight as approximately 40% of the weight is due to the presence of the spermatozoa and testicular fluid secretion (both interstitial and seminiferous tubule fluid) (Brooks, 1979; Karkun *et al.*, 1974; Robaire *et al.*, 1985).

The vas deferens connects the epididymis to the urethra. The epithelium lining the vas deferens is complex and has been shown to perform both secretory and absorptive functions. In humans, at least four different cell types have been identified: principal, pencil, mitochondrion-rich and basal cells (Setchell *et al.*, 1994). The epithelium of the human vas deferens is surrounded by a thick muscle layer which is composed of three coats, an inner longitudinal layer, a circular or oblique layer and an outer longitudinal layer. In the rat vas deferens there is a progressive decrease in the complexity of the epithelial and muscle layers as the organ becomes more distal to the epididymis.

1.3.6 The Accessory Glands

The accessory glands comprise the prostate, seminal vesicles, ampulla and the bulbourethral gland (Cowpers Gland). The prostate gland is a lobular organ which is present in all mammalian species. It is defined as a compound tubuloalveolar gland and can be identified as either disseminate or discrete. If the glandular acini remain within the lamina propria without penetrating the urethral muscle it is defined as disseminate but when the gland forms a definite body outside the urethral muscle it is termed discrete (Setchell *et al.*, 1994). The rat prostate comprises the ventral prostate (a bilobed structure next to the urethra) and a dorsolateral lobe. The coagulating gland is an additional lobe of the prostate which is also known as the anterior prostate. The lobes of the prostate drain into the urethra through multiple ducts. The seminal vesicles are a pair of glands which lie next to the prostate. They are not present in all animals but do exist in both man and rats. The glands are composed of compact tissue arranged in multiple lobes which contain a system of secretory ducts. It is lined with a pseudostratified epithelium which has a row of round basal cells and a row of larger columnar cells. The remainder of the gland comprises two layers of connective tissue with a layer of smooth muscle sandwiched between (Setchell *et al.*, 1994).The ampulla is a spindle-shaped thickening

at the terminal region of the ductus deferens. It is not present in all animals but is present in man. Bulbuorethral glands (Cowpers glands) are multilobular, compound tubuloalveolar glands which are present in most mammals. They are located near the bulb of the penis and are connected to the urethra by a duct. The alveoli of the ducts are lined with mucous-like cells and the ducts by a single layer of cuboidal/squamous cells (Setchell *et al.*, 1994).

1.4 Fluids of the Male Reproductive System

All of the cells within the testis, epididymal duct system and accessory organs are bathed in a tissue specific fluid creating specific environments for the immature and maturing spermatozoa. This fluid varies in composition in the different regions of the male reproductive tract and provides a continuously changing environment for the developing germ cells in the testis and for their maturation in the epididymis.

1.4.1 Interstitial Fluid of the Testis

The outside of the seminiferous tubules and the surrounding interstitial tissue are bathed by interstitial fluid (IF). Within the testis the capillaries have a high vascular permeability with no apparent restriction to the passage of proteins into IF (Setchell *et al.*, 1994) and the protein concentration of IF is similar to blood plasma. The volume of IF is reduced in prepubertal rats after hypophysectomy and oestradiol treatment (Widmark *et al.*, 1987).

Interstitial fluid is collected by making an incision in an isolated testis and letting the fluid drip into a container (Setchell *et al.*, 1994). The drip technique demonstrated almost 10 times higher levels of androgen binding protein (ABP) compared with testicular venous blood (Turner *et al.*, 1984) and about 100 times as much arginine vassopressin (AVP) (Pomerantz *et al.*, 1988). This technique, found IF high in potassium and testosterone in comparison to blood plasma but other techniques, such as using an ion-sensitive microelectrode to measure ion concentrations directly in the testis of an anaesthestised rat, found no difference in potassium concentrations between IF and plasma (Setchell *et al.*, 1994). Measurements of the concentrations of ions and proteins in IF will vary widely depending on the method used to both collect samples and measure a parameter.

1.4.2 Seminiferous Tubule Fluid (STF)

The Sertoli cells of the testis secrete seminiferous tubule fluid (STF). This secretion is probably a constitutive function of the Sertoli cell but it is only evident after the development of Sertoli cell junctions and the formation of a lumen within the seminiferous tubules (Sharpe, 1994). These changes within the seminiferous tubule occur around puberty and prior to the

onset of the first meiotic division during the first wave of spermatogenesis. It is at this time that the Sertoli cells cease replicating and undergo several maturational changes. One of these changes involves the formation of tight junctions between adjacent Sertoli cells which is often referred to as the blood-testis-barrier. This barrier serves at least two functions. First, it allows the formation of an adluminal compartment within the seminiferous tubule allowing the maintenance of a separate, controlled environment around the meiotic and post meiotic germ cells. Secondly, as postmeoitic cells are haploid, they would be detected as 'foreign' by the body's immune system, the barrier prevents the entry of immune cells (i.e. macrophages) which could detect haploid-specific antigens. In rats, a barrier to the entry of macromolecules was noted in the majority of tubules on postnatal days 15 and 16 and was completely formed in all tubules prior to postnatal day 18 attaining adult characteristics by day 22 postnatal (Russell *et al.*, 1989).

The appearance of the Sertoli cell barrier occurs at around the same time as lumen formation within the seminiferous tubule. Sertoli cells can secrete STF both apically and basally, although the bulk is secreted apically (Setchell *et al.*, 1978), (Setchell, 1978). The barrier channels the secreted STF and presumably causes a rise in hydrostatic pressure due to the increased fluid secretion and induces lumen formation. STF flows down the seminiferous tubules driven by the fluid pressure caused by secretion and/or the contraction of the peritubular myoid cells (Clermont, 1958). In rats, some developing seminiferous cords show lumen formation as early as postnatal day 10 and lumen diameter increases slowly until day 30 and then rapidly until day 70 (Jégou *et al.*, 1983; Russell *et al.*, 1989; Setchell, 1970).

Efferent duct ligation has been used to detect STF production in neonatal animals (Jégou *et al.*, 1982). By this technique, STF was not detected prior to day 15 postnatal and rose rapidly after day 20 coincident with the prepubertal rise in serum FSH. Rats at 25 days of age were administered a subcutaneous injection of either FSH, LH, testosterone propionate, hCG (human chorionic gonadotrophin) or prolactin at the time of efferent duct ligation. A significant rise in STF was only observed after FSH treatment in neonatal animals (Jégou *et al.*, 1982). Around puberty, the high increase in testosterone production appears to make many testicular functions more responsive to testosterone and less responsive to FSH. In the adult rat, the production of STF is thought to be controlled by testosterone levels and the presence of elongate spermatids (Sharpe, 1994). Efferent duct ligation was used in adult rats to examine STF production before and after hypophysectomy (Jégou *et al.*, 1983). Forty eight hours after hypophysectomy there was a significant (26%) fall in STF production, and by 16-44 days, STF production was at 15% of control animals. Another set of animals were

treated four days after hypophsectomy for 3 days with either LH, FSH, testosterone propionate, or FSH plus testosterone propionate (Jégou *et al.*, 1983). STF production was restored in rats treated with LH, testosterone propionate or testosterone propionate administered with FSH (Jégou *et al.*, 1983). When these treatments were administered to intact rats only LH significantly increased STF production (testosterone propionate failed to reach significance). These results suggest that in the adult rat STF production is regulated by testosterone secreted by the Leydig cells in response to stimulation by LH (Jégou *et al.*, 1983).

The rate of STF secretion (as measured by electrophysiology) is 21µl/hour/testis in adult rats (Setchell, 1970). The rate of fluid secretion can be reduced by cooling the testis or removing glucose and potassium from the bathing medium (Setchell *et al.*, 1994). The formation of STF has been proposed to occur by several mechanisms. Setchell (1969), proposed that solutes are pumped by Sertoli cells from the interstitial fluid into the intercellular spaces above the occluding junctions of the seminiferous epithelium. This would cause a hypertonic fluid in the intercellular spaces and fluid would be drawn in to restore osmolarity. Fluid would then flow along the intercellular spaces into the seminiferous tubule lumen (Setchell, 1969). This hypothesis remains unproven. Another mechanism of STF formation was put forward by Waites and Gladwell (Waites and Gladwell, 1982). Their hypothesis was based on the identification of Na^+/K^+-ATPase pumps within the basal membrane of the Sertoli cell. Sodium ions were proposed to diffuse into the Sertoli cell and were pumped back into the interstitium and exchanged for a K^+ which would diffuse into the lumen. Bicarbonate ions are also thought to be involved as carbonic anhydrase inhibition decreases the secretion of STF. The movement of ions would induce the formation of an osmotic gradient and water would be drawn into the seminiferous tubules. Sertoli cells cultured *in vitro* have been shown to secrete over 100 proteins into STF but less than 30 have been identified (Bardin *et al.*, 1994). These can be classed as transport binding proteins (androgen binding protein, sulphated gycoprotein-2, transferrin, ceruloplasmin), proteases (cyclic protein-2, plasminogen activator) hormones (inhibin B, activin) and energy metabolites (lactate, pyruvate and α-keto acids).

Androgen binding protein (ABP) is a heterodimeric glycoprotein with a molecular weight of 85-90 KDa. It is comprised of two subunits H (47 KDa) and L (41 KDa), which have been shown to be present in the isolated native molecule in a ratio of 3:1 (Larrea *et al.*, 1981). ABP has a high affinity for both testosterone and dihydrotestosterone and it is regarded as a carrier protein for these steroids. ABP is secreted bidirectionally but 67% is secreted apically into the lumen of the seminiferous tubules and transported through the rete testis, efferent ducts

and into the epididymis (Bardin *et al.*, 1994) Immunocytochemical methods demonstrate that ABP is endocytosed in the efferent ducts and the proximal region of the epididymis (Bardin *et al.*, 1994).

After the initiation of spermatogenesis, STF bathes the developing germ cells and transports the released spermatozoa to the epididymis. STF forms the basis of the fluid which bathes the spermatozoa throughout their maturation and passage through the rete testis, efferent ducts, epididymis and vas deferens. The composition of this fluid is constantly modified by these various regions of the excurrent duct system (presumably to provide an optimal environment for maturation and storage). It is important to understand the composition of this fluid as it is may give us some insight into what are the important factors required for germ cell development. Also, by comparing fluids from different regions of the excurrent duct system the ions, proteins, or hormones that have been resorbed or secreted in a particular region can be determined. This will aid understanding of the functions of these tissues and the process of sperm maturation. The alterations in ion and protein composition between the fluids of the seminiferous tubules, rete testis and cauda epididymis are compared in Section 1.4.5.

1.4.3 Rete Testis Fluid (RTF)

Rete testis fluid (RTF) has a different ionic and protein composition from that of STF (Setchell *et al.*, 1994). RTF is much easier to sample than STF and can be collected using catheters either chronically implanted in large animals (rams, bulls, boars) or acutely in small animals (rats, rabbits, wallabies) (Setchell *et al.*, 1994). The fluid is a suspension of immature spermatozoa at a concentration of ~30 million/ml in rats (Setchell *et al.*, 1994). The ionic composition is different from blood plasma, testicular interstitial fluid and STF (see Tables 1 and 2) and it contains more chloride and less bicarbonate than any of the other fluids. Rete testis fluid contains very little protein (~1mg/ml) suggesting that it may be resorbed by the epithelial cells of the rete testis (Hinton and Keefer, 1983). The level of androgen binding protein is similar to that of STF, however the level of transferrin is about one third of the level in STF but this is still almost 75 times the level detected in blood plasma (Sylvester and Griswold, 1984; Turner *et al.*, 1984). Serum albumin comprises between 11-17% of RTF protein (Skinner *et al.*, 1987). The level of testosterone gradually decreases between interstitial fluid (150 ±27 ng/ml), STF (91 ±14ng/ml) and RTF (33 ±3ng/ml) but the level of the metabolite DHT is higher in RTF (Comhaire and Vermeulen, 1976) (Turner *et al.*, 1984).

1.4.3.1 Endocytosis in the Rete Testis of the Rat

Determining whether the rete testis epithelium is capable of endocytosis and/or exocytosis would aid understanding of how the differences in the fluid composition between STF and RTF was achieved. The endocytotic functions of the epithelial cells has been determined by examining ultrastructural changes using electron microscopy after the injection of electron dense tracers. Using CF and HRP Morales et al.(1984) demonstrated that both fluid phase and absorptive endocytotic pathways are active in the rete testis epithelium. Binding of CF was observed at the apical plasma membrane and in underlying vesicles 1-5 minutes after injection. Within 1-2 hours some tracers were internalised into lysosomes while other tracers were transported through the cells and found in intercellular spaces or at the base of the cells (Morales *et al.*, 1984).

1.4.4 Efferent Duct Fluid

RTF passes through the efferent ducts into the epididymis. This creates the initial source of epididymal luminal fluid. The efferent ducts are known to be the major site of fluid resorption in the excurrent ducts. Winet (1980) proposed a mathematical model which predicts that the flow of fluid through the efferent ducts is based on a cilio-peristaltic model. The main contributor inducing fluid movement is peristaltic action while the cilia induce reflux and reduce the flow rate (Winet, 1980). The mechanism of fluid resorption within the efferent ducts has often been inferred from studies carried out on the caput epididymis as this region is easier to cannulate. Whether the same mechanisms of fluid resorption are working at both these sites remains to be determined but there are proteins which are expressed in the efferent ducts that are involved in fluid uptake which are not present in the epididymis, e.g. Aquaporin-1 (Brown *et al.*, 1993). In the caput epididymis, fluid resorption is energy-dependent and can be modified by hormones (Setchell *et al.*, 1994).

Efferent duct fluid can be examined by *in vivo* micropuncture and microperfusion. These techniques are used to compare the fluid composition between the rete testis and epididymis to determine the changes which occur on transit through the efferent ducts. Between the rete testis and the end of the efferent ducts in the rat, spermatozoa are concentrated by a factor of 25 fold from 2.24 $x10^4/\mu l$ to 6.00 x $10^5/\mu l$, indicating that 96.2% of the fluid entering the efferent ducts is reabsorbed (Clulow *et al.*, 1994). Microperfusion studies have demonstrated that the rate of fluid resorption is dependent on luminal flow rate (Clulow *et al.*, 1996). The principal solutes in luminal fluid are Na^+ (137-144mM), Cl^- (113-130mM) and, K^+(13.5 ± 2.4 mM) (Clulow *et al.*, 1994; Man *et al.*, 1997). Osmotic pressure did not vary along the ducts or differ significantly from blood plasma (301-307 mosmol/kg) (Clulow *et al.*, 1994) or

rete testis fluid (311.2 ±1.7) (Man *et al.*, 1997). This demonstrates that the efferent ducts reabsorb the majority of the testicular output of water and inorganic electrolytes, and most of the protein. It is the epididymis, rather than the efferent ducts which is responsible for the accumulation of specific organic compounds such as inositol (Clulow *et al.*, 1994).

1.4.4.1 Ion and Water Transport

One of the major functions reported for the efferent ducts is fluid resorption. The exact mechanisms involved in fluid resorption are not clearly understood. Two main proposals have been suggested to explain fluid resorption. One is that it occurs secondary to ion transport via the action of the Na/K-ATPase. An ATP-dependent Na^+/K^+ pump has been immunolocalised to the rat testis, efferent ducts and epididymis (Ilio and Hess, 1992). The staining pattern was found to be most intense in the basolateral region of the cells but there was no immunostaining reported on the apical plasma membranes. Studies that have used a specific inhibitor of the Na-pump (ouabain) decreased fluid resorption by 50%. This suggests that this an important mechanism, but not the only route involved in fluid resorption from the efferent ducts. Castration has also been shown to inhibit Na^+ and water transport but this effect was reversed after the administration of testosterone. This suggests the process is steroid dependent. The other hypothesis is that fluid is taken into the cells by endocytosis which would allow water to enter the cell along with ions and other macromolecules. Water could be used by the cell in reactions with carbonic anhydrase or in other processes, while the remainder exits the cell by an unknown mechanism (Hamilton, 1975). In 1993, a water channel protein, CHannel forming Integral membrane Protein of 28 KDa (CHIP28), subsequently renamed Aquaporin-1, was localised to the efferent duct epithelium (Brown *et al.*, 1993). This channel may also prove to be important in fluid resorption across the efferent duct epithelium.

Various studies have used different techniques to explore the function of the efferent ducts by determining what enzymes and receptors they express. The efferent ducts have been found to be rich in carbonic anhydrase, Na^+/K^+-ATPase and various steroid receptors. Carbonic anhydrase has been localised to the efferent ducts of the rat and bull (Cohen *et al.*, 1976; Goyal *et al.*, 1980). The secretion of hydrogen ions causes the pH of a solution to be lowered as occurs in the stomach; conversely in the pancreas the secretion of bicarbonate causes the fluid to become more alkaline (Ilio and Hess, 1994). The pH of the fluid within the efferent ducts changes as it passes through to the epididymis. In fact, between the rete testis and the caput epididymis the pH of efferent duct luminal fluid becomes substantially more acidic (lowering from pH 7.2 - 6.4) suggesting that carbonic anhydrase is one component

used to induce luminal acidification within the efferent ducts by the production of hydrogen ions (Levine and Marsh, 1971).

1.4.4.2 Does the Renin-Angiotensin System (RAS) regulate fluid and electrolyte balance in the efferent ducts?

There have been many reports that components of the renin-angiotensin system (RAS) are present in different regions of the male reproductive tract. This system will be briefly reviewed along with the evidence supporting its role in male reproduction and with particular attention to evidence surrounding its involvement in efferent duct fluid resorption.

The renin-angiotensin pathway is active in the kidney and regulates water and electrolyte balance. One of the major players in this system is aldosterone which is a mineralocorticoid secreted by the zona glomerulosa of the adrenal cortex. Aldosterone acts on the distal convoluted tubule of the nephron when blood pressure is low and induces the juxtaglomerular cells of the kidney to secrete renin. Renin is an enzyme which converts angiotensinogen (a component of the α_2-globulin fraction of plasma proteins) to the decapeptide angiotensin I in the circulation. A second enzyme, angiotensin converting enzyme, cleaves two amino acids from the carboxy terminus of angiotensin I to produce angiotensin II, an active octapeptide hormone (Vinson *et al.*, 1995). Angiotensin II causes constriction of the efferent arterioles that lead away from the glomerulus and increase blood pressure. Angiotensin II can also stimulate the production of aldosterone from the adrenal gland. Aldosterone increases the resorption of water and Na^+ by the distal convoluted tubules and collecting tubules. The resorption of Na^+ into the peritubular blood induces the movement of Cl^- and HCO_3 to maintain the electrostatic balance. High levels of K^+ in extracellular fluid have also been shown to induce aldosterone secretion by the adrenals (Tortora and Anagnostakos, 1990).

The efferent ducts have been studied to determine whether fluid and electrolyte resorption is controlled by adrenal mineralocorticoids (Man *et al.*, 1997). Testicular fluid output and fluid flow from the efferent ducts were determined in rats ten days after adrenalectomy ± aldosterone administration or in sham operated controls. Fluid output was also assessed in rats given the aldosterone antagonist spironolactone for ten days. In sham operated rats testicular fluid output was 36.0 ±7.8 µl/h and fluid flow from the efferent ducts was 1.23 ±0.12 µl/h. This resulted in a fluid resorption of 94.8% of the testicular output by the efferent ducts. None of the treatments altered the rate of testicular output or the rate of fluid resorption nor did they alter the osmolality or electrolyte concentrations. Like the homologous proximal

tubule of the kidney, fluid resorption in the efferent ducts is independent of adrenal mineralocorticoid control (Man *et al.*, 1997).

Exactly what role/s the components of the renin-angiotensin system play in male reproductive physiology is unclear. Renin (prorenin) mRNA has been isolated from rat and mouse testes and localised to the Leydig cells in rats and humans (Vinson, *et al.*, 1997). Angiotensin Converting Enzyme (ACE) has been localised to the testis and efferent ducts which are considered sites of angiotensin I synthesis (Holbrugger *et al.*, 1982). The ACE isozyme in the testis is found in Leydig cells and developing spermatozoa and has also been located in the epididymis (Vinson *et al.*, 1997). Ligand binding studies and autoradiographic studies have revealed Angiotensin II receptors in Leydig cells and evidence indicates that angiotensin II may be involved in regulating androgen secretion as it inhibits cAMP and testosterone responses to human hCG (Vinson *et al.*, 1997). In females, angiotensin II has been shown to stimulate ovarian oestrogen production (Speth and Husain, 1988) and treatment with an angiotensin II antagonist (saralasin) can inhibit ovulation in perfused rat ovaries (Peterson *et al.*, 1993). Functional receptors for Angiotensin II have been localised to the epididymis and spermatozoa (rat and human) but their role within the male reproductive system is not clear (Grove and Speth, 1989). Some authors have suggested that they may be important factors in the regulation of sperm motility (Vinson *et al.*, 1995), spermiogenesis and the contraction of the epididymal smooth muscle cells (Ilio and Hess, 1994), while others suggest they are involved in electrolyte and fluid flux across the epithelium (Vinson *et al.*, 1997; Wong and Uchenda, 1990).

1.4.5 Epididymal Fluid

Many studies have shown that there are large differences in the concentrations of inorganic and organic compounds between epididymal luminal fluid and blood, supporting the hypothesis of a functional blood-epididymal barrier (Crabo and Gustafsson, 1964). The luminal compartment is composed of water, ions, small organic molecules, proteins, glycoproteins and spermatozoa. The composition of this fluid is constantly changing as it travels through the excurrent duct system. Specific proteins have been shown to be endocytosed from the epididymal lumen. ABP has been localised to the cytoplasm of principal cells within the efferent ducts and proximal segment of the caput epididymis (Attramadal *et al.*, 1981; Pelliniemi *et al.*, 1981). Other proteins such as α-2 macroglobulin and transferrin are internalised by receptor-mediated endocytosis (Robaire and Hermo, 1988). Many ions and small molecules have also been shown to be secreted by the cells of the epididymal epithelium. Potassium and phosphate are secreted, as are carnitine, inositol and α-

glycerylphosphorylcholoine. Micropuncture studies have demonstrated that the level of sodium decreases between the efferent ducts and caput epididymis. Similarly, the level of chloride falls between the caput and cauda whereas the concentration of potassium increases in the luminal fluid in all regions of the epididymis (Jenkins *et al.*, 1980). Ion transport across the epididymal epithelium is thought to be dependent on the presence of testosterone as is the synthesis and secretion of epididymal glycoproteins (Wong and Yeung, 1978). Micropuncture has also been used to measure the pH of the luminal fluid and showed that there was a gradual luminal acidification on passage through the epididymis (Levine and Marsh, 1971). The pH of seminiferous tubule fluid was found to be pH 7.31 and this was reduced to 6.48 in the distal caput epididymis (Levine and Marsh, 1971). Luminal acidification indicates the existence of ion channel proteins in the epididymal epithelial membranes. Two apical proteins, Na/H exchanger and H^+-ATPase have been implicated in luminal acidification. The immunoexpression of these two proteins in the efferent ducts of control and neonatally oestrogen treated animals will be addressed in Chapter 6.

1.4.6 Comparison of Fluid Compositions in Compartments of the Male Reproductive Tract

The following tables illustrate how the ionic composition (Table 1.1) and the concentration of low molecular weight organic compounds (Table 1.2) changes along the reproductive tract in STF, RTF and fluid sampled from the cauda epididymis.

Table 1.1 The Ionic Composition(mM) of Fluids of the Rat Male Reproductive System

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HPO_4^{2-}	HCO_3^-
STF	108 ±7	50 ±5	0.44 ±0.02	1.2 ±0.18	120 ±5	<0.1	20
RTF	143 ±4	14 ±1	0.81 ±0.09	0.39 ±0.09	140 ±2	<0.1	21
Cauda	26 (15-37)	47 (43-55)	0.18 (0.11-0.25)	1.1 (0.9-1.3)	24 (20-27)	13 (9.5-16)	6.7

Data from (Setchell *et al.*, 1994). The values are shown are means with either ± the standard error margin or with the range of values shown in parentheses.

Table 1.1 illustrates the increasing concentration of both Na^+ and Ca^{2+} from STF to RTF whereas the concentrations of K^+ and Mg^{2+} decrease. The levels of Cl^-, HPO_4^{2-}, HCO_3^- remain unchanged between these two fluids. Fluid analysed form the cauda epididymis shows large reductions in Na^+, Cl^- and Ca^{2+} while HPO_4^{2-} and HCO_3^- are increased. Both K^+ and Mg^{2+} show levels similar to those recorded for STF.

Table 1.2 The Concentration of Low Molecular Weight Organic Compounds and Total Protein(mg/ml) in Fluids of the Rat Reproductive System

	Glucose	Inostitol	Carnitine	Glycero-phosphocholine	Phospho-choline	Protein
STF	<1	1.8	<1	<0.1	<0.1	6
RTF	<0.1	2.5	<1	<0.1	<0.1	
Cauda		31	54	32	20	32

Data from (Setchell *et al.*, 1994).

The concentration of low molecular weight organic compounds also varies throughout the fluids of the reproductive system (Table 1.2). The level of inositol is greatly increased between STF and the fluid of the cauda epididymis as are carnitine, glycerophosphocholine, phosphocholine and total protein levels. Understanding how these changes in ions and proteins are controlled would aid understanding of both epididymal function and the process of sperm maturation.

1.5 Physiological Control of the Male Reproductive System

The physiological control of reproduction is maintained at many different levels. The reproductive system is part of the endocrine system and has control centres within the hypothalamus and pituitary gland (hypothalamo-pituitary axis) which includes a classical negative feedback system. There are also local control loops involving paracrine and autocrine signals which maintain organ/cell homeostasis, especially within the testis. The major hormones that control reproduction are Gonadotrophin Releasing Hormone (GnRH), Luteinising Hormone (LH), Follicle Stimulating Hormone (FSH), testosterone and inhibin. GnRH is secreted from the terminals of hypothalamic GnRH neurons and interacts with specific GnRH receptors in the gonadotroph cells of the pituitary. The gonadotrophs release two hormones, FSH and LH, which are both important in regulating gonadal function. LH acts via specific receptors on the interstitial Leydig cells of the testis to stimulate production of the steroid, testosterone. FSH acts on specific receptors on Sertoli cells and there is some debate as to whether spermatogonia also have specific receptors for FSH (Baccetti *et al.*, 1998; Rannikko *et al.*, 1996). One of the products induced after FSH stimulation of the Sertoli cell is inhibin B (Byrd *et al.*, 1998).

The importance of the gonadotrophic hormones is evident after the administration of a GnRH antagonist. Spermatogenesis is suppressed when the release of LH and FSH is inhibited. This suggests also that non-gonadotrophic hormones are unlikely to be able to maintain spermatogenesis in mammals (Weinbauer and Nieschlag, 1993). However, there are receptors for other pituitary hormones within the testis. Receptors have been identified for prolactin (PRL), growth hormone (GH), thyroid stimulating hormone (TSH) and ACTH. Both PRL and GH can increase the number of LH binding sites on Leydig cells. The mechanisms of action of testosterone and FSH on spermatogenesis are not known.

Testosterone is synthesised by the Leydig cells from cholesterol or acetyl coenzyme A and it diffuses into the interstitial fluid and blood stream. As the blood circulates the increased level of testosterone is detected at the level of the hypothalamus which inhibits the secretion of

GnRH from the hypothalamus and subsequently reduces LH release from the pituitary. The levels of LH secreted into the blood stream will also be detected by the hypothalamus and influence the level of GnRH released by the hypothalamus. Once the level of testosterone falls beneath a certain level the release of GnRH by the hypothalamus is no longer inhibited. In a similar way, the other gonadotrophin, FSH is also controlled by a negative feedback loop. FSH acts via specific receptors on Sertoli cells within the seminiferous tubules of the testis resulting in increased secretion of inhibin B which is important in the negative feedback regulation of FSH at the pituitary level. Increased levels of inhibin in the circulation are responsible for decreasing the level of FSH released by the anterior pituitary. This feedback loop is shown diagrammatically in Figure 1.6.

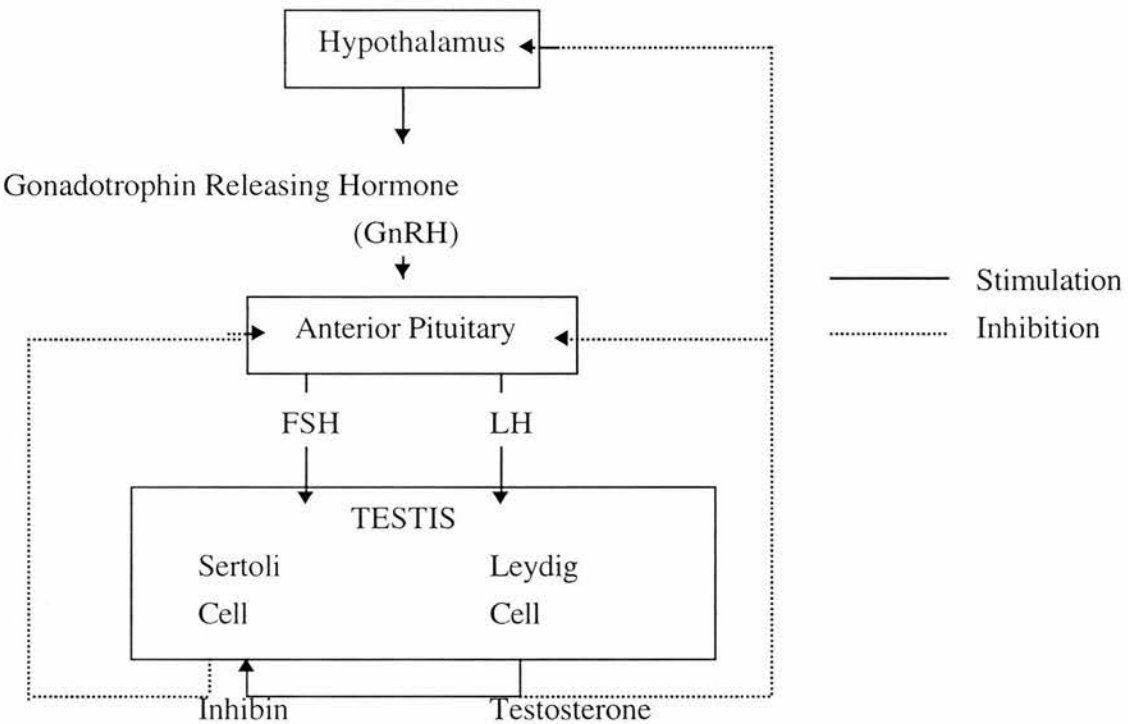

Figure 6 Demonstrating the Levels of Physiological Control Within the Reproductive Axis

Modified from (Tortora and Anagnostakos, 1990).

1.5.1 The Hypothalamus

The physiological control of reproduction is influenced by the central nervous system (CNS), particularly by the secretions of the hypothalamus. The hypothalamus is a small region at the base of the brain and lies immediately above the pituitary gland. The third ventricle separates the hypothalamus into two symmetrical halves. The hypothalamus contains many small areas

with dense aggregations of neurons called *hypothalamic nuclei* (Johnson and Everitt, 1995). These nuclei are involved in the hypothalamic regulation of many diverse functions such as sexual behaviour, thermoregulation, ingestive behaviour and integration of autonomic activity (Johnson and Everitt, 1995). The centres involved in the control of reproduction are the *supraoptic, paraventricular, arcuate, ventromedial* and *suprachiasmatic* nuclei. These hypothalamic regions have either a direct neural or an indirect vascular link to the pituitary gland. The posterior pituitary secretes hormones (vasopressin and oxytocin) directly into the systemic circulation from axon terminals arising from large magnocellular neurons (Riskind and Martin, 1989). The cell bodies of these neurons arise in the supraoptic and paraventricular nuclei and their axons project directly into the posterior pituitary. In contrast, the hypothalamus communicates with the anterior pituitary via a vascular connection. The hypothalamus secretes releasing (and release inhibiting) hormones into a specialized portal system via the axon terminals of small parvocellular neurons. These neurons terminate in the precapillary space of the median eminence (infundibulum) (Riskind and Martin, 1989). The portal system arises at the base of the median eminence in a vascular band that is continuous with the pituitary stalk (Riskind and Martin, 1989). Hypothalamic releasing hormones are secreted into this closed portal system to reach the anterior pituitary.

1.5.1.1 The Hypothalamic Hypophysiotrophic Hormones

The hypothalamus secretes many hormones important in endocrine control (e.g. thyroid-releasing hormone, corticotrophin-releasing hormone) but the major hormone which controls reproduction is Gonadotrophin Releasing Hormone (GnRH). GnRH is secreted by the parvocellular neurosecretory cells of the hypothalamus into the portal system of the pituitary. It then interacts with GnRH receptors on the surface of the gonadotroph cell to stimulate gonadotropin synthesis and release and stimulate target gene expression. GnRH is a decapeptide with a short half-life of between 5 and 7 minutes (Lincoln, 1989). The protein is not synthesized in its mature form but as a precursor molecule 92 amino acids long. This protein comprises a signal sequence which joins directly with the active hormone. GnRH (and subsequently LH) are secreted in pulses at the rate of about one an hour thus they are termed circhoral pulses. The pulse generator resides in the arcuate nucleus of the mediobasal hypothalamus (Johnson and Everitt, 1995). This pulsatile release is essential for the secretion of FSH and LH. Continual stimulation with GnRH would result in continuous occupancy of the GnRH receptor sites which would lead to their desensitization and internalisation by the gonadotroph leading to the down regulation of gonadotrophin secretion (Johnson and Everitt, 1995).

1.5.2 The Pituitary Gland

The pituitary lies outside the brain directly beneath the hypothalamus. It sits in the hypophyseal fossa of the sphenoid bone overlain by a fold of dura mater (diaphragma sellae) which has a central opening through which the pituitary stalk runs. The pituitary gland has three lobes, the anterior lobe (adenohypophysis), the posteior lobe (neurohypophysis) and the intermediate lobe which is a small division of the anterior pituitary. The anterior pituitary is the region that houses gonadotroph cells which respond to GnRH, therefore this region will be discussed further.

1.5.2.1 The Anterior Pituitary

The anterior pituitary comprises glandular epithelial cells which synthesize and package hormones into secretory vesicles that are then released into nearby fenestrated capillaries (Page, 1988). The pituitary is composed of several different cell types which are specialised for the secretion of different hormones: lactotrophs (secrete prolactin), thyrotrophs (secrete thyroid stimulating hormone), gonadotrophs (secrete Follicle Stimulating Hormone (FSH) and Luteinsing Hormone (LH), somatotrophs (secrete growth hormone) and corticotrophs (secrete adrenocorticotropic hormone (ACTH) and melanocyte stimulating hormone). The gonadotrophs are the major players controlling the reproductive axis and secrete their hormones in response to GnRH pulses from the hypothalamus. The majority of the gonadotrophs synthesize both LH and FSH whilst about 20% of the cells secrete either FSH or LH but not both. The hormones are glycoproteins composed of an α and β subunit. The α chain is identical between TSH, LH, hCG and FSH, but the β chain is hormone specific. Once secreted into the systemic circulation the gonadotrophins act at their target organs, the gonads, to induce biological responses, including the production of sex steroids. In the male, LH acts on Leydig cells within the interstitium of the testes to induce the production of testosterone. Testosterone performs many biological functions within the testis and excurrent duct system and also acts as a feedback molecule to regulate the secretion of GnRH and LH as shown in Figure 1.6. Levels of FSH can also be partially altered by testosterone levels but a more complete suppression is induced by the Sertoli cell product inhibin B (Pierik *et al.*, 1998).

1.5.3 The Testicular Feedback Hormones Inhibin B: a useful marker of spermatogenesis?

Inhibins are polypeptide glycoproteins which are related to the TGF-β family and have been implicated in the control of spermatogenesis by exerting negative feedback on FSH secretion (Byrd *et al.*, 1998) or perhaps via paracrine effects within the testis. Inhibin is composed of an α-subunit which is linked by disulphide bridges to one of two highly homologous β-

subunits (A and B). This determines the formation of either inhibin A (α-βA) or inhibin B (α-βB). The β-subunits show some homology with the TGF-β superfamily (Halvorson and DeCherney, 1996).

In humans in the immediate neonatal period there is an increase in hypothalamic-pituitary function which results in a transient increase in gonadotrophin levels and gonadal steroid production. These levels decline over the next few years (Halvorson and DeCherney, 1996). The trend is mirrored by the level of inhibin B. In male infants, serum levels of inhibin B (inhibin A levels are not measurable) are in the adult male range and decrease again until puberty (Anderson *et al.*, 1998). Inhibin levels subsequently rise progressively, roughly corresponding to the stage of puberty and increases in sex steroid production (Halvorson and DeCherney, 1996). A similar trend in production of inhibin B has recently been observed in the rat (Sharpe *et al.*, 1999). The neonatal increase in inhibin B in rats and humans coincides with the period of Sertoli cell replication. Decreasing the number of Sertoli cells via administration of a GnRH antagonist causes corresponding reductions in both inhibin B and FSH (Sharpe *et al.*, 1999).

Prior to puberty in humans, Sertoli cells produce both the alpha and beta subunits of inhibin. A recent study has described that during puberty the expression of the β-B subunit changes to germ cells whilst Sertoli cells retain the expression of the α-subunit (Anderson *et al.*, 1998). This might suggest that both Sertoli cells and germ cells (pachetyne spermatocytes to early spermatids) contribute to inhibin B secretion. These studies require further confirmation but subject to this, in the future, inhibin B could become a useful marker of the activity of the seminiferous tubules and the efficiency of spermatogenesis (Anderson *et al.*, 1998; Pierik *et al.*, 1998).

1.5.4 Testosterone

Testosterone is essential for the maintenance of spermatogenesis but how it carries out this role is not understood. In the testis, testosterone is synthesized and secreted by Leydig cells. Once synthesized via the steroidogenic pathway it rapidly leaves the cell and enters the interstitial fluid (Cooke *et al.*, 1972). From here, testosterone can enter either the seminiferous tubules or the circulation. The amount of testosterone leaving via the bloodstream is dependent on blood flow (Galil and Setchell, 1987). The concentration of testosterone within the testis is far higher than the levels within the circulation. The most acurate measurement of intratesticular testosterone levels can be achieved by measuring the level of testosterone in testicular venous blood and this level is reduced by 50% as blood passes up the spermatic

cord through the pampiniform plexus (Maddocks and Sharpe, 1989). By the time the blood has reached the level of the posterior vena cava the testosterone levels have been reduced by more than 90% (Maddocks and Sharpe, 1989). The concentration of testosterone in the spermatic vein is about 250 times more concentrated than levels detected in peripheral venous serum (Maddocks and Setchell, 1989). Other androgens are also synthesised by the testis and can also enter the circulation (e.g. androstanediol, androstenedione, dehydroepiandrosterone, and dihydrotestosterone). The combined concentration of all these other androgens is less than 15% of the total concentration of testosterone in the spermatic vein and the contribution of steroids in the blood stream from other steroid producing sources, such as the adrenal account for less than 5% of the total serum testosterone. Of the testosterone in the circulation, 98% is bound to serum proteins such as sex hormone binding globulin (SHBG), leaving only 2% free.

The potent androgen 5-α dihydrotestosterone (DHT) is also synthesised in the testis and other regions of the excurrent duct system. DHT is formed from testosterone by the enzyme 5-α reductase. It is 10 times more potent than testosterone and has a 2-fold greater affinity for the AR as it forms more stable complex on binding, has a low plasma concentration and binds tightly to plasma proteins. (Grino *et al.*, 1990). Testosterone acts by binding to intracellular androgen receptors which are present in Leydig cells, peritubular myoid cells and Sertoli cells of the adult testis. Studies localising AR have shown that expression within the Sertoli cell is not constant throughout the spermatogenic cycle and AR expression increases in Sertoli cells during puberty implying that the role of AR in Sertoli cells is more important after puberty than neonatally (Sharpe, 1994). Similarly, the level of the FSH receptor varies between different spermatogenic stages but binding studies and mRNA analysis studies give contradictory results as to which stages display the highest expression and as to whether FSH receptors are present on spermatogonia (Kliesch *et al.*, 1992).

The production of STF is under the control of testosterone in the adult rat whereas prepubertally it is under the control of FSH (Au *et al.*, 1986; Free *et al.*, 1980; Jégou *et al.*, 1982; Jégou *et al.*, 1983). Similar changes are probably true for the secretion of androgen binding protein and the control of Sertoli cell functions. The Sertoli cell becomes increasingly less responsive to FSH during puberty and into adulthood (Sharpe, 1994). During the spermatogenic process, testosterone appears to play a major role in the development or progression of cells at stages VII-VIII of the spermatogenic cycle of the rat as germ cells at these stages show increased rates of degeneration when testosterone is removed (Dym and Madhaw Raj, 1977; Flickinger, 1977; Russell *et al.*, 1981). The cyclical changes in Sertoli

cells observed during the spermatogenic process are probably manifested by testosterone acting indirectly (i.e. via the Sertoli cells) on the appropriate germ cell complements (Sharpe, 1994).

1.6 Fetal / Neonatal Development of the Testis and Associated Duct Systems

The testis develops along the ventral cranial portion of the mesonephros (Byskov and Hoyer, 1994). The initial stage of gonad formation occurs when the primordial germ cells migrate from the endodermal yolk sac through the hindgut to the coelomic epithelium and the underlying mesenchyme of the mesonephros. At this time, cells of mesonephric origin are released and migrate to the coelomic epithelium. This induces the establishment of cell lineages to support the migrating germ cells (Byskov and Hoyer, 1994).

1.6.1 The Mesonephros

The mesonephros is part of the developing nephric structures. There are three nephric structures which arise consecutively during mammalian fetal development, these are termed the pro-, meso, and metanephros (Byskov and Hoyer, 1994). All of these structures arise from the nephrogenic cord. The pronephros never functions as a kidney in mammals but serves as an inducer for the development of the meso- and metanephros (Du Bois, 1969). The nephrons of the mesonephros develop from the nephrogenic cord and form a connection with the pronephric duct or Wolffian duct. In humans, the mesonephros does act as a kidney with functional glomeruli but in the mouse the glomeruli are not functional (Grinsted and Aagesen, 1984). The developing gonads remain connected to the mesonephros through cell streams attached to the adjacent duct systems (Wolffian and Müllerian). These cell streams develop into the rete testis or rete ovarii (Byskov and Hoyer, 1994). Connection with the mesonephros is required for normal gonad development, as when ovaries are removed from the mesonephros their differentiation and germ cell development are inhibited (Byskov, 1974).

1.6.2 Coelomic Epithelium

The sexually indifferent gonad consists of coelomic epithelium (germinal epithelium) underneath which lies mesenchymal tissue. Early in gonadal development the coelomic epithelium is invaded by capillaries and nervous tissue (Pelliniemi, 1975). In males, it is delimited by a basal lamina which separates the developing testicular cords. Also the epithelium and the outermost testicular cords are separated by mesenchymal tissue which becomes the tunica albuginea (Byskov and Hoyer, 1994). An early event in testicular

differentiation is the development of a close association between germ cells and somatic cells (Sertoli cells) leading to the formation of testicular cords. The presumptive Sertoli cells express the gene *Sry* and initiate testicular differentiation. The Y chromosome contains a region containing the testis determining gene *Sry* (Swain and Lovell-Badge, 1999). In mouse development *Sry* is expressed in the genital ridge in males at day 10.5-11pc to initiate testis development. Expression of *Sry* occurs in a wave from anterior to posterior where it is thought to trigger the formation of Sertoli cells. Sertoli cells in turn then direct the differentiation of the other cell types in the testis (Swain and Lovell-Badge, 1999). *Sry* is probably involved in the switching on of Müllerian Inhibiting Substance (MIS, also termed Anti-Müllerian Hormone (AMH)) by fetal Sertoli cells which then orchestrate male development. On day 17.5 post coitum the fetal rat testis consists of cords that are simple arches perpendicular to the axis of the testis connecting to the rete testis (Byskov and Hoyer, 1994). The connection with the rete testis ensures communication with the mesonephric cells and cells migrate in and contribute to the Sertoli cell, peritubular myoid and Leydig cell populations. After testicular cord formation the testis rounds up and there is only a thin attachment with the mesonephric tissue (Byskov and Hoyer, 1994).

After the germ cells (prespermatogonia) have been enclosed in the testicular cords they enter rounds of mitosis. This creates clusters of cells connected via cytoplasmic bridges which divide in synchrony. The developing Sertoli cells originate from the coelomic epithelium but mesonephric cells are also known to contribute to this cell population (Byskov, 1986). Sertoli cells divide during fetal and neonatal life but this ceases before the onset of spermatogenesis (Clermont and Perey, 1957; Nagy, 1972). As the testis develops, the prespermatogonia move from a central to a peripheral location within the seminiferous cords and the Sertoli cell shape becomes more irregular with cytoplasmic extensions. The Sertoli cells develop desmosomes, then incomplete tight junctions and small gap junctions (Byskov and Hoyer, 1994). The steroidogenic capacity of the fetal testis is not well mapped temporally. Androgen secretion has been detected in fetal guinea pig testes prior to the differentiation of Leydig cells (Byskov and Hoyer, 1994). Sertoli cells are known to be responsible for the secretion of MIS which induces the regression of the female anlage, the Müllerian duct (Lovell-Badge, 1992). The initial secretion of MIS may be switched on by *Sry* (Lovell-Badge, 1992).

Testicular cord formation precedes Leydig cell differentiation and the Sertoli cell is thought to drive the formation of the fetal Leydig cell population. Leydig cells divide slowly in fetal life but a new generation ('adult Leydig cells') differentiate rapidly during puberty (Vergouwen *et al.*, 1991). The fetal and adult populations of Leydig cells display different characteristics.

Fetal Leydig cells are found in clumps which are not observed after day 10 in the neonatal rat and testosterone secretion by the fetal testis is closely correlated with the cytodifferentiation and an increase in volume and number of the Leydig cells (Picon, 1976; Roosen-Runge and Anderson, 1959). Leydig cells contain many active steroidogenic enzymes and are capable of synthesising both androgens and oestrogens.

1.6.3 Formation and Differentiation of the Genital Ducts

The undifferentiated gonad is the same structure in either sex and follows one of two distinct developmental pathways. However, two sets of internal ducts develop in both sexes as distinct and separate structures termed, Müllerian and Wolffain ducts. The two duct systems lie side-by-side and differentiation depends on gonadal sex. In females, maintenance of the Müllerian duct is required for normal reproductive tract development (fallopian tube, oviduct, uterus and upper vagina) while in males, testosterone is required for the stabilisation and development of the Wolffian duct into the epididymis, vas deferens and seminal vesicles. Prior to the onset of virilsation the female (Müllerian) duct has to regress. Sertoli cells secrete MIS which induces the regression of this duct. In females neither of these hormones are produced. The lack of MIS prevents regression of the Müllerian duct and the lack of testosterone prevents the stabilization of the Wolffian duct and therefore it regresses. Female development is often referred to as the default pathway.

1.6.4 The Undifferentiated Ducts

As mentioned above two ductal systems arise independently. The Wolffian duct develops from the excretory duct of the mesonephros and is recognisable before gonad formation whilst the Müllerian (paramesonephric) duct develops along the back of the Wolffian duct in a cranial-caudal direction at the time the gonad forms (Dohr and Tarmann, 1984). Both of these duct systems are straight tubules lined with a simple epithelium with cells ranging from squamous to cylindrical and their differentiation is dependent on epithelial-mesenchyme interactions. The mesenchyme is probably the target of hormones and growth factors which then drive the morphogenesis of the epithelium (Cunha *et al.*, 1981).

1.6.4.1 Differentiation of the Wolffian Duct and Urogenital Sinus

The cranial portion of the Wolffian duct develops into the epididymis, the central portion becomes the vas deferens and the caudal region forms the seminal vesicles (Byskov and Hoyer, 1994). The rete testis is connected to the Wolffian duct via mesonephric tubules which develop into the efferent ducts and join the epididymis. The differentiating epididymis grows in length and becomes highly convoluted. The epithelium is columnar and has many

apical microvilli. In humans the epithelium is actively secretory during the third trimester of pregnancy (Zondek and Zondek, 1965). The developing duct becomes surrounded by a layer of mesenchyme which develops into a layer of smooth muscle cells. This clearly differentiates the epididymis from the vas deferens as the latter develops a thick layer of trilaminar muscle (Byskov and Hoyer, 1994).

It is well cited in the literature that testosterone is responsible for the stabilisation of the Wolffian duct and subsequent development of the excurrent ducts, and that DHT is reponsible for prostate growth and the masculinization of the external genitalia. Recent evidence regarding the times and sites of expression of the enzyme 5α-reductase suggest this interpretation is an over simplification. It is true that during the initial stages of sex differentiation the synthesis of DHT from testosterone by 5α-reductase does not occur in the Wolffian duct of embryonic rats, rabbits, guinea pigs or humans, but high levels of 5α-reductase are observed at later periods (Tsuji *et al.*, 1994).

5α-reductase is undetectable during the stabilisation of the Wolffian duct but it is elevated at the times when the duct is differentiating into the epididymis, vas deferens and seminal vesicles (Wilson and Lasnitzki, 1971), (Siiteri and Wilson, 1974). The structures that develop from the urogenital sinus are known to be dependent on DHT since patients with 5α-reductase deficiency show poor prostatic development and feminized external genitalia but the Wolffian duct derivatives develop normally (Wilson, 1992). However, in serum free organ culture growth and branching morphogenesis of the neonatal mouse seminal vesicle (Wolffian derived) is dependent on DHT not testosterone (Shima *et al.*, 1990). Furthermore, growth of the cranial regions of the Wolffian duct can be induced by either DHT or testosterone *in vitro* (Tsuji *et al.*, 1991) and *in vivo* (Schultz and Wilson, 1974). At day 14.5 post coitum (pc) the different regions of the male reproductive tract are producing various levels of functional 5-α reductase. The level of functional 5α-reductase was inferred from the concentration of ^{3}H 5-α-reduced steroids produced from ^{3}H testosterone over the 48h culture period (Tsuji *et al.*, 1994). After the culture period the fetal testis showed little 5α-reductase activity. The cranial region of the Wolffian duct (prospective epididymis) had very low 5α-reductase activity supporting the involvement of testosterone in epididymal development. The level of 5α-reductase activity gradually increased in a cranial to caudal direction with the developing Wolffian duct. The region which develops into the seminal vesicles had a higher 5α-reductase activity than did the developing urogenital sinus at day 14.5 - 16.5 pc.

Exactly how testosterone and dihydrotestosterone induce the differentiation of the male reproductive tract is unclear but it undoubtedly involves strong mesenchyme / epithelial cell interactions. Some studies have shown that prostaglandin E_2 is able to masculinize the genital duct of a fetal mouse in *in vitro* organ cultures and also that Wolffian duct differentiation could be inhibited by the addition of an antibody against this prostaglandin (Gupta, 1989; Gupta and Bentlejewski, 1992). This suggests that one mediator of testosterone action could be prostaglandin E_2. The secretion of paracrine factors from the mesenchyme around the developing seminal vesicles and prostate, such as keratinocyte growth factor, play an important role in seminal vesicle development and in the growth of both normal and neoplastic prostate epithelial cells (Tsuji *et al.*, 1994).

1.7 Steroids and the Male Reproductive System

Androgens and oestrogens act within the same tissues of the male reproductive system and often within the same cells. Oestrogen action is the main concern of this thesis and will be emphasised here, but the ratio of androgens: oestrogens is likely to be important in oestrogen action within the male reproductive system, so the sites of androgen action will also be reviewed (Section 1.7.4).

In order to determine the physiological role of oestrogen in male reproduction it would be logical to establish where oestrogens act. This can be assessed by determining where radio-labeled oestrogen binds, where oestrogen receptors are localised or by determining the consequences of disrupting the genes involved in either oestrogen synthesis or action. This section will examine the literature concerning where oestrogens act in the male and the consequences of abnormal oestrogen action.

1.7.1 Oestrogen Receptor Expression In the Male

Oestrogens and other steroid hormones act by diffusing into the cell cytoplasm and interacting with specific protein receptors which then dimerise and translocate into the nucleus. Once in the nucleus, the steroid receptors bind to target DNA and activate or repress gene transcription. Prior to the identification and sequencing of the oestroegn receptor (ERα) there were no antibodies available, therefore studies used indirect methods of detection such as steroid autoradiography to determine where oestrogen (or DES) bound or measurement of the binding coefficients of oestrogen binding proteins collected from the cytosol after sucrose gradient density centrifugation. There were a number of conflicting reports within the literature as to where oestrogen acted in the male reproductive tract. Some studies suggested that only the efferent ducts expressed ERs while others reported ERs throughout the male

reproductive tract. These controversies were clarified in 1996 when a novel form of the oestrogen receptor (ERβ) was cloned (Kuiper *et al.*, 1996). This explained why immunocytochemical studies which only detected ERα produced different results from steroid autoradiography with labeled oestrogen which detected all oestrogen binding sites. The historical data regarding oestrogen binding will be reviewed here. The new data regarding ERβ will be introduced in Chapter 3.

The majority of studies have been performed in the mouse and a few studies have examined fetal expression. Stumpf *et al.*, (1980) injected tritiated DES into pregnant mice on day 16 of gestation and examined binding in the fetus by autoradiography (Stumpf *et al.*, 1980). They reported that binding was concentrated in the cell nuclei of certain hypothalamic and extra-hypothalamic nuclei of the brain, the anterior pituitary, laryngeal mesenchyme, the mesenchyme surrounding both the Müllerian and Wolffian ducts, the urogenital sinus, rectum, mammary gland, gubernaculum testis and the skin (Stumpf *et al.*, 1980). Cooke *et al.* (1991) examined ER expression in the male fetal mouse reproductive tract from day 16 of gestation to 10 days postnatal. They found that all fetal reproductive organs expressed mesenchymal ER (efferent ducts, urogenital sinus, Wolffian duct and epididymis, vas deferens, seminal vesicles, coagulating glands, prostate and bulbuorethral glands) (Cooke *et al.*, 1991b). The efferent ducts were the first site of expression in the developing male tract, with epithelial ER expression present on fetal day 16 and thereafter. The epididymis did not express ER until fetal day 19. There appeared to be a gradient in epididymal ER expression as the epithelial cells of the efferent ducts contained 3 times as many silver grains as the more distal regions of the epididymis (Cooke *et al.*, 1991b). By postnatal day 6 the epithelium of the seminal vesicle and coagulating gland both expressed ER but the epithelia of the vas deferens, prostate and bulbourethral glands were never ER positive (Cooke *et al.*, 1991b).

An immunocytochemical study was performed on fetal male mice in 1992 (Greco *et al.*, 1992). At fetal day 13, ER was detected in both the epithelial and mesenchymal cells of the Wolffian duct. By day 15, the testis was clearly differentiated and staining was present within the testicular cords and interstitium. The staining within the fetal duct system was identical to that on day 13 but was judged to be more intense. On fetal day 17, Leydig cells and possibly peritubular myoid cells showed positive staining but cells within the seminiferous cords showed little staining (Greco *et al.*, 1992). Another imunocytochemical study examined expression of ERα from late fetal life through to adulthood in the rat (Fisher *et al.*, 1997). In the fetal, neonatal and adult rat testis, only Leydig cells were immunopositive. No immunostaining was observed within the seminiferous cords/tubules. ERα was found in the

mesenchyme surrounding the mesonephric tubules but postnatally it was expressed within the epithelium of the rete testis with the most intense staining occurring in the efferent ducts (Fisher *et al.*, 1997). Except for sporadic staining within the epididymis during neonatal life the epididymal duct was negative for ERα (Fisher *et al.*, 1997). A study conducted in adult macaques determined that the efferent ducts contained a similar amount of ER per mg DNA as did the oestrogen-treated endometrium and oviducts (West and Brenner, 1990). In adult goats the only site of ER expression detected by immunocytochemistry was the nonciliated cells of the efferent ducts (Goyal *et al.*, 1997).

The sites of oestrogen action within the epididymis have also been explored by autoradiography after the administration of tritiated oestradiol (Schleicher *et al.*, 1984). This study found the highest number of grains in the efferent ducts, initial and caput segments of the epididymis. In the epididymis there was a higher labeling of clear cell nuclei with oestradiol than with DHT, suggesting that oestradiol may be important in modulating clear cell function (Schleicher *et al.*, 1984).

These studies demonstrate the conflicting opinions about sites of oestrogen action within the male reproductive system. The majority of studies do however agree that the efferent ducts of the epididymis are the major site of oestrogen action within the male reproductive tract.

1.7.1.1 Oestrogen Receptor Mutations

Early in the 1990s, functional ERs were considered essential for embryonic survival as they were regarded as essential for implantation and no naturally occurring mutations in the ER had ever been clinically described. In 1993, however, an oestrogen receptor minus mutant mouse (ERKO) was described (Lubahn *et al.*, 1993). Both sexes were viable and phenotypically normal (Lubahn *et al.*, 1993) but they were both sterile (Korach, 1994) suggesting that oestrogen is essential for normal fertility. Further examination of the male mice showed ERKO mice had a higher mean testis weight but 3 times lower daily sperm production and with a higher number of abnormal sperm in cauda epididymal fluid than in either heterozygotes or wild type mice (Lubahn *et al.*, 1996). In young adult mice (3-5 months) spermatogenesis was occurring in some seminiferous tubules while others were fluid filled with dilated lumens and a disorganised seminiferous epithelium; still others had only Sertoli cells present (Eddy *et al.*, 1996). The seminiferous tubule lumens were dilated by day 20 as was the rete testis which was also abnormally extended into the testis (Eddy *et al.*, 1996).

A transgenic mouse has also been created which overexpresses estrogen receptor-α (Davis *et al.*, 1994). This mouse displayed normal differentiation of sexual organs of both sexes and there were no major fertility problems observed in male mice (Davis *et al.*, 1994). Transgenic female mice overexpressing ERα displayed prolonged gestation which led to a loss of litters due to difficulties in parturition (Davis *et al.*, 1994).

A year after the ERKO mouse had been reported the first clinical report of a patient with a mutated ER was published (Smith *et al.*, 1994). The man was 28 years old, very tall and had incomplete epiphyseal closure and reduced bone density (3.1 SD below age matched mean). He underwent puberty normally but his fertility status is unknown. He had elevated serum oestradiol and oestrone levels but normal serum testosterone. He also had an impaired glucose tolerance with hyperinsulinaemia (Smith *et al.*, 1994). Oestrogen is essential for both the development and maintenance of optimal bone mass in both sexes and this effect is mediated by ERα (Kusec *et al.*, 1998). Oestrogen has been shown to have effects on the chondrocytes involved in longitudinal growth in epiphyseal growth centres (Kusec *et al.*, 1998).

1.7.2 Aromatase Expression in the Male Reproductive tract

Another method for assessing the importance of oestrogen in male reproductive physiology is to examine the sites of aromatase expression. Aromatase is the P450 enzyme responsible for the conversion of certain androgens into oestrogens. There are very few studies that have characterized the expression of aromatase within the male reproductive tract. Within the testis, Sertoli cells of immature males produce oestrogen and aromatase expression is highly responsive to FSH before puberty (Rosselli and Skinner, 1992). Leydig cells are regarded as the source of aromatase in the adult testis (Dorrington *et al.*, 1978). Within the epididymis, aromatase was localised to the clear cells of the corpus and caput epididymis (Janulis *et al.*, 1996b). The most interesting location of aromatase is in developing spermatozoa both in the testis and the epididymis. Within the seminiferous tubules, round and elongating spermatids have been shown by *in situ* hybridisation and immunoctyochemistry to express aromatase (Nitta *et al.*, 1993) (Janulis *et al.*, 1996a). As sperm transit through the epididymis the level of aromatase decreases (Janulis *et al.*, 1998). This suggests that spermatozoa are a source of oestrogen during their transit through the excurrent duct system. This oestrogen could interact with ER within the epithelia to alter their maturational environment.

A couple of clinical reports have been described for men with mutations in aromatase. The clinical phenotype is very similar to the patient described above with a mutation in the ERα

gene. Both cases showed decreased bone density, incomplete epiphyseal closure and increased serum FSH. One patient had macro-orchidism while the other was oligospermic (but his other siblings were also oligospermic) (Morishima *et al.*, 1995; Qin *et al.*, 1996).

1.7.3 Animal Models - the effects of synthetic oestrogen exposure

The effects of potent oestrogens such as DES, oestradiol benzoate and ethinyl oestradiol have been studied in animal models. The effects observed appear to depend on the dose, time and duration of exposure. Oestrogen could act at several levels within the male reproductive tract. Oestrogen affects specific neuronal regions in the brain that are important for sexual behaviour, they modulate gonadotrophin secretion from the pituitary gland, or they can directly affect the reproductive tract. Oestrogen receptors are present in all of these sites but there are also reports in the literature suggesting that steroids can interact with cell surface receptors to induce changes that do not require RNA synthesis (Sadler and Maller, 1982). Whether all of these interactions are occurring in the developing gonadal system is not clear.

Administration of sex steroids during perinatal life can have permanent or long lasting effects in the central and peripheral mechanisms controlling reproduction. Masculinization of the brain occurs in early neonatal life and is dependent on testicular secretions (Arai *et al.*, 1983). Removal of the testis results in "feminine males" which secrete gonadotrophins in a cyclical pattern and display feminine sexual behaviour. In contrast, the female brain can be masculinized by the neonatal administration of oestrogen or androgen (which is thought to be aromatised to oestrogen) (Arai *et al.*, 1983). This is known to occur in the sexually dimorphic nuclei of the preoptic area which is markedly larger in females but can be altered by perinatal steroid manipulation (Arai *et al.*, 1983). Sexual dimorphism has also been identified in the hypothalamic arcuate nucleus and the medial amygdaloid nucleus. These changes induce altered sexual behaviour which in some studies is the cause of infertility.

In rodents detrimental effects on the reproductive tract can be induced with either pre- or early postnatal injections of oestrogens. Studies have examined the administration of DES to time mated pregnant mice over days 9-16 gestation (100μg/kg maternal body weight) as this covers the development of the reproductive tract (McLachlan and Newbold, 1975). The fertility of the male offspring was assessed at 7 months of age, 6/10 males housed separately with two fertile females failed to impregnate the mice. At 9-10 months the reproductive structures were assessed post mortem. Testicular abnormalities were recorded in 15/24 animals with six of these having at least one intra-abdominal testis. DES also appeared to induce the appearance of multinucleate giant cells within the seminiferous epithelium of scrotal testes. Eight mice

had epididymal cysts and six of these had testicular lesions. The accessory glands also displayed several abnormalities such as distension, inflammation and ulcers (McLachlan and Newbold, 1975).

The major effects induced in animals after DES exposure are abnormal testes with disordered spermatogenesis, an increased incidence of cryptorchidism, poor semen quality and infertility (McLachlan, 1981). Oestrogen administration during development appears to interfere with normal development as males show delayed or incomplete regression of the Müllerian duct (McLachlan, 1981); this has been verified by *in vitro* organ culture experiments (Newbold *et al.*, 1984). Oestrogen receptors have been identified in the Müllerian ducts at the time of regression (Greco *et al.*, 1993).

Postnatal treatment of rats with β-oestradiol-3-benzoate or clomiphene citrate from birth delayed the formation of the blood-testis-barrier which is normally formed between 16-19 days until day 24 postnatal (Vitale *et al.*, 1973). When testicular histology was assessed at days 30 and 60 both compounds induced the appearance of large lumens within the seminiferous tubules with many exfoliated germ cells present in the luminal fluid. The authors judged the effects of oestradiol benzoate to be more severe than those induced after the administration of clomiphene citrate (Vitale *et al.*, 1973). Testicular abnormalities were also noted in rats which were given a single injection of oestradiol benzoate on postnatal day 5 (Kincl *et al.*, 1963). Rats received an injection of either 120, 30 or 10 μg. When treated rats were housed with fertile females only the rats which had received the 10μg dose sired any litters (11/13males) (Kincl *et al.*, 1963). When assessed in adulthood, the rats which were administered 120μg oestradiol benzoate had testis weights which were reduced by more than 50% and the seminiferous tubules demonstrated almost a complete loss of the seminiferous epithelium except for a group of large tubules in the central region where the seminiferous epithelium was preserved, but immature germ cells were present in the lumen. The lower doses of oestradiol benzoate did not induce testicular atrophy and testicular morphology appeared normal (Kincl *et al.*, 1963).

Many studies investigating neonatal oestrogen exposure report bilateral or unilateral cryptorchidism which may be related to the underdevelopment or lack of the gubernaculum testis which is thought to aid testicular descent into the scrotum (Arai *et al.*, 1983). Aside from cryptorchidism, testicular abnormalities are often described including those already mentioned and interstitial cell hyperplasia and tumours, Sertoli cell hyperplasia and Sertoli cell carcinoma in situ (Arai *et al.*, 1983). Lesions have also been reported within the epididymis.

Dunn and Green (1963) reported lesions of the epididymis in more than half the male mice sampled at 13-16 months after a single injection of 0.1ml of a 2% DES suspension on the day of birth. Both single and multiple cysts on both epididymides were noted (Dunn and Green, 1963). Warner et al., (1979) reported that epididymal cysts occurred in 70% of mice treated from days 1-5 postnatal with 50μg oestradiol (Warner *et al.*, 1979). Similar findings have been frequently observed after DES exposure (Arai *et al.*, 1983). Cyst formation is thought to be related to the persistence of the Müllerian duct structures as there is a close association with increases in cyst formation after exposure to DES (McLachlan, 1981).

1.7.4 Androgens and the Reproductive System

The expression of androgen receptors within the rat fetal testis has been determined by immunocytochemistry (Majdic *et al.*, 1995). On fetal day 16.5 AR was present in the mesenchyme surrounding the Wolffian duct but was negative around the Müllerian duct. A day later, staining was evident within the epithelium of the Wolffian duct. Within the testis staining was confined to cells of the interstitium and in particular, to the peritubular myoid cells. These cells did not co-localise with cells expressing 3β-hydroxysteroid dehydrogenase and so were not considered to be Leydig cells (Majdic *et al.*, 1995). Sertoli cell staining only became evident a week after birth (Majdic *et al.*, 1995). An earlier study had used tritiated DHT to examine AR binding sites by autoradiography during fetal and early postnatal life of the male mouse (Cooke *et al.*, 1991a). They found that the mesonephric tubules, Wolffian duct, urogenital sinus and their derivatives all had AR in mesenchymal/stromal cells. The first site of epithelial androgen receptor expression was the efferent ducts from fetal day 16 onwards. The expression of AR occurred in a cranial - caudal direction and the vas deferens showed epithelial AR expression on fetal day 19 (Cooke *et al.*, 1991a).

In adult rats, AR expression was immunolocalised to Leydig cells and peritubular myoid cells of the interstitium (Bremner *et al.*, 1994). Within the seminiferous tubules the Sertoli cells displayed a stage-dependent pattern of expression. Nuclear staining increased throughout stages II-VII and declined during stage VIII and was barely detectable within stages IX-XIII (Bremner *et al.*, 1994). In the adult ram epididymis, AR have been immunolocalised to the principal cells of the epididymis. The region between the central caput and the proximal corpus shows the most intense AR staining and is therefore determined to be the most androgen-dependent (Tekpetey *et al.*, 1989).

1.7.4.1 Expression of 5α-Reductase

As the androgen receptor can bind either testosterone or DHT it is important to know the sites of synthesis for the enzyme 4-ene steroid 5α-reductase (5α-reductase) as it catalyses the formation of this hormone from testosterone.

There are two forms of 5α-reductase (Type 1 and 2). Type 2 5α-reductase is found in most male reproductive tissues and has 10-15 times higher affinity for testosterone than does type 1. Type 2 has been located to the testis, epididymis and vas deferens, while type 1 was shown to be more abundant in the seminal vesicles and ventral prostate (Normington and Russell, 1992). Both mRNAs follow a gradient of expression with enzyme levels being higher in the initial segment of the epididymis and decreasing towards the cauda. This gradient is set up and maintained during fetal and early neonatal life (Tsuji *et al.*, 1994). Most 5α-reductase activity in the epididymis is due to Type 2 activity in view of its acidic pH optimum and its activity at low substrate concentrations (Normington and Russell, 1992). Gene defects in 5α-reductase type 2 lead to pseudohermaphroditism in humans. These 46, XY males have male internal urogenital ducts as testosterone acts to stabilize the Wolffian duct but have female external genitalia as DHT is required for the formation of the male external genitalia (Andersson *et al.*, 1991). Inactivating mutations of the AR gene results in various degrees of defective sexual differentiation. At the most severe the complete lack of androgen action prevents the development of both the Wolffian duct and the external genitalia so the child appears female but is infertile. As the presence of a testis induces the secretion of MIS the female ductal system also regresses. The testes generally remain abdominally and are usually removed due to the fear of malignancy (MacLean *et al.*, 1995).

1.8 Conclusions

With the reported increases in disorders of male reproductive health and analogous changes in wildlife it seems plausible to consider that some environmental factor(s) might be having a detrimental effect on reproductive tract development. There are stark similarities between the changes observed to be increasing in humans and those induced in the sons of mothers exposed to the potent non-steroidal oestrogen DES *in utero*. This suggests that substances that can act like an oestrogen may be involved in the increasing incidence of abnormalities. Similarly, as many of the conditions are present at birth or have links with events occurring *in utero* or neonatally, it suggests that the causative agents may be acting at these sensitive time points. The role of oestrogen in male reproductive physiology is not understood nor is its role in fetal development. These basic questions regarding the physiological role(s) of

oestrogens have to be answered to understand how exogenous oestrogens may disrupt normal male reproductive tract development.

The experimental work undertaken in this thesis focuses mainly on the efferent ducts of the male reproductive tract as they have been highlighted as a major target of oestrogen action. The level of ERα within the efferent ducts is higher than that of the oestrogenised uterus and the discovery of another ER isoform (ERβ) will identify other sites of oestrogen action which had not been previously considered. The efferent ducts have been shown to be abnormal in ERKO mice which are also infertile suggesting that oestrogen may be important for normal fertility. The major function of the efferent ducts is in fluid resorption, therefore it is important to discover whether any steps of this process are regulated or can be disrupted by oestrogens.

The experimental chapters of this thesis will be presented as follows; chapter 3 addresses the sites of oestrogen receptor-β immunoexpression in the marmoset monkey thus fully reviewing the sites of oestrogen action within the male reproductive tract. Chapter 4 focuses on a possible mechanism of fluid resorption within the efferent ducts by immunolocalising Aquaporin-1 from fetal/neonatal life to adulthood in both the rat and marmoset monkey. The remainder of the experimental chapters focus on the effects of oestrogen and 'environmental oestrogens' on the male reproductive tract. Chapter 5 examines the consequences of neonatal oestrogen treatment on the histology of the rete testis. Chapter 6 focuses on the effects induced in the efferent ducts by examining AQP-1 immunolocalisation, protein levels, epithelial cell height and general histology. Chapter 7 focuses more specifically on the epididymis and localisation of potentially oestrogen regulated ion channel proteins. This chapter first assesses the possibility that CFTR may be modulated by oestrogen. Secondly, this chapter compares the localisation of two ion channels (Na/H exchanger and H^+-ATPase) before and after neonatal oestrogen exposure to determine whether any markers of epididymal function have been altered. Finally, Chapter 8 summarises the main findings and conclusions of the experimental work and suggests several avenues which might lead to further the present understanding of oestrogen action within the male reproductive system.

Chapter 2 Materials and Methods

2.1 Animals and Animal Welfare

The animals used in these studies were maintained and treated in accordance with Government guidelines as stated in the Animals Scientific Procedures Act, 1986. Most of the animals used in these studies were male Wistar rats which were bred and maintained at the MRC Reproductive Biology Unit animal facility. All rats were maintained under standard conditions of 12 hour light / 12 hour dark cycle and in an ambient temperature of 21°C. Male animals from late fetal life through to adulthood were assessed at various time points. As treatment regimens were implemented from early neonatal life the female pups were removed from the litters after birth and cross fostered. The male pups were housed with their mothers until death or weaning on day 22 days. After this time the male pups were housed together and fed food and water *ad libitium*. Animals that were not undergoing any further procedures were killed by CO_2 inhalation followed by cervical dislocation in accordance with Schedule 1. Very young neonatal animals (i.e. younger than 18 days) were killed by CO_2 inhalation followed by decapitation. Similarly, all fetuses were decapitated after removal from the mother.

This study also used tissue obtained from marmoset monkeys. All of this tissue was acquired from tissue stored within the department which arose from other studies. The author is thankful to Dr Hamish Fraser for the use of this tissue.

2.2 Treatment Regimes and Test Compounds

These studies examine the effects of administering potent oestrogens (DES, ethinyl oestradiol) and many weakly oestrogenic environmental chemicals. These treatment regimes involved subcutaneous administration on alternate days from days 2-12 postnatal (inclusive) or every day until day 18 or weaning. At times this produced a hectic injection schedule so the help of Drs Richard Sharpe and Katie Turner is duly noted.

2.2.1 Diethylstilboestrol (DES)

DES is a well known potent synthetic oestrogen which is known to mimic the action of oestrogen *in vivo*. This compound was used as the principal positive control treatment to assess the effects of high dose oestrogen. Cohorts of animals were set up and administered DES (Sigma, Poole, Dorset, UK) at doses of 10μg, 1μg or 0.1 μg in 20μL of corn oil. The neonatal rats were subjected to sub-cutaneous injections on alternate days from day 2-12 inclusive (i.e. six injections).

2.2.2 Ethinyl Oestradiol (EE)

The treatment regime for EE (Sigma) was identical to that for DES except that only one dose (10μg/20μL corn oil) was administered to neonatal rats using the same injection schedule.

2.2.3 Gonadotrophin Releasing Hormone Antagonist (GnRHa)

GnRHa was administered to separate any specific actions of the oestrogenic compounds from those which occur through oestrogens acting in a negative feedback loop to reduce the amount of gonadotrophins released from the pituitary. The administration of GnRHa effectively abolishes gonadotrophin secretion and any effects observed in these animals which also occurred in animals treated with oestrogen could therefore be a consequence of the reduced gonadotrophin levels rather than of any specific, directs effect of oestrogen administration. GnRHa was to some degree used as a negative control treatment.

GnRHa (Antarelix, Europeptides. Argenteuil, France) was administered via subcutaneous injection at 10μg/kg in 20μL 5% mannitol on postnatal days 2 and 5. The dose administered was based on previous studies which had demonstrated complete suppression of FSH levels beyond day 18 (Sharpe *et al.*, 1998; Sharpe *et al.*, 1999).

2.2.4 Bisphenol A

Bisphenol A (Aldrich Chemicals Limited, Dorset, UK) was administered at a dose of 0.5mg in 20 μL corn oil on postnatal days 2-18 (inclusive). This dose was based on the maximum solubility obtained by dissolving this chemical in oil.

2.2.5 Octylphenol

Octylphenol was administered at a dose of 2mg/20μL corn oil administered on days 2-18 inclusive. Again, this dose was chosen due to the maximum solubility of this compound in oil.

2.2.6 Methyl-parabens

Methyl-parabens was a gift from Dr J. Ashby (Zeneca CTL, Cheshire, UK), and was injected at a dose of ~2mg/kg/day in 2ml/kg corn oil on days 2-18 inclusive; animals that were killed on day 18 were sampled 4 hours after they received their final injection. The dose was based on recent studies which assessed its effects *in vivo* on rat uterine weight (Routledge *et al.*, 1998).

2.2.7 Genistein

Genistein, which is one of the isoflavenoid phytoestrogens found in soya, was administered between days 2-18 inclusive at a dose of ~4mg/kg/day in 2mL/kg vehicle (phosphate buffered saline (PBS) + 2.5mg/mL gelatin. Genistein was initially dissolved in 0.4M KOH and diluted 1:20 with PBS producing a dosing concentration of 800μg/mL. The mothers of rats treated neonatally with genistein and their respective controls (treated with the appropriate vehicle) were fed on a casein-based soy-free diet (B.S.&S., Edinburgh, Scotland). Rats were killed on days 10 and 18, while they were undergoing treatment. Rats were killed 4 hours after their daily injection. The dose of genistein administered was based on figures reported by Setchell *et al.* (Setchell *et al.*, 1997) which showed this was the total isoflavenoid intake by human 4-month old infants fed on 100% soy formula milk diet (Setchell *et al.*, 1997).

2.2.8 Tamoxifen

Tamoxifen (Sigma) was administered at a dose of ~2mg/kg in 20μl corn oil on days 2-16 inclusive. This dose was based on previous studies which reported significant effects of this dose on male reproductive development.

2.3 Methods for Tissue Fixation, Processing and Staining

2.3.1 Tissue Fixation for Frozen Sections

A cohort of 25 day old animals were treated to be used in studies performed in the Renal Unit, Harvard Medical School. This laboratory only had access to a cryotome therefore a different fixation protocol was employed. The testis with the epididymis attached was removed and fixed in 4% paraformaldehyde in 0.1M sodium phosphate buffer for 6 hours and washed for 30mins in 0.2M ammonium chloride to quench the aldehyde and stop the fixation process. The tissue was stored in 0.1M sodium phosphate containing 0.02% sodium azide and stored at 4°C.

2.3.2 Tissue Preparation for Frozen Sections

The tissue was rinsed and incubated in PBS containing 30% sucrose for several hours/ overnight until the tissue was saturated and sank to the bottom of the vial. Sucrose penetration of the tissue acts as a cryopreservative and helps prevent ice crystals forming once the tissue is frozen. The epididymis was removed from the testis and mounted onto a cutting chuck. The tissue was covered in OTC compound 4583 (Tissue-Tek, Miles, Inc.) and slowly frozen to -30°C in a Reichert-Jung Fridgocut 2800 cryostat. Cryosections (5μm) were cut with high profile disposable knives. Tissue sections were mounted onto Fisher Superfrost Plus microscope slides (Fisher Scientific, Los Angeles, CA, USA). These tissue sections were stored at -20°C until used for immunostaining.

2.3.3 Tissue fixation for Paraffin Embedding

In animals which were less than 25 days old the testis and epididymis were removed together and placed in Bouin's' fixative (500ml 40% v/v formaldehyde, 100ml acetic acid and 2 litres saturated picric acid) for 5 hours and then transferred into 70% ethanol until processed into paraffin wax. Animals older than 25 days were perfusion fixed via the dorsal aorta. This procedure was performed by Dr Sharpe and is described in Millar *et* al., (Millar *et al.*, 1994). The rats were anaesthetised with halothane prior to perfusion. Once anaesthetised, the abdominal cavity was exposed and a catheter inserted into the dorsal aorta. Normal saline was perfused for approximately 3 min until the testicular blood vessels cleared completely. Filtered Bouin's' fluid was then perfused for 45 min. The testis and epididymis were removed together. The cranial region of the testis which contains the rete testis was cut off whilst still attached to the epididymis. The corpus and cauda portions of the epididymis were removed and the remaining piece of testis attached to the caput epididymis was postfixed for a further 5h before being transferred into 70% ethanol.

Prior to being processed, the tissue was orientated to ensure that tissue cross sections through the testis, rete testis and efferent ducts could be achieved. In animals 18 days and older this meant cutting off the cranial region of the testis attached to the caput epididymis and removing the corpus and cauda epididymis. The cranial portion of testis was then bisected along the plane of the epididymis to cut the rete testis and efferent ducts into two equal halves.

2.3.4 Tissue Processing and Sectioning for Paraffin Blocks

Tissue was dehydrated through a series of graded alcohols before being saturated by paraffin wax. This process was performed using a 17.5h automated cycle on a Leica TP-1050 processor (Leica UK Limited, Milton Keynes, UK). The tissue was then embedded with the cut surfaces facing up so that the rete testis and efferent duct region could be sectioned. Glass microscope slides to be used for immunocytochemistry were dipped twice in a 4% v/v solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone (BDH), washed in acetone and rinsed in double distilled water and dried overnight. The TESPA coating increased the adherence of the tissue to the slide. Slides used for *in situ hybridisation* were purchased to ensure they were RNase free and coated with poly-L- lysine, again to promote tissue adherence to the slide. Paraffin sections were cut (5μm) using a hand-operated microtome (Jung RM2035; Leica) with disposable blades. Sections were floated onto a heated water bath (containing distilled water for immunocytochemistry sections or RNase free water for sections to be used for *in situ hybridisation*) at approximately 50°C. The sections were transferred onto slides and dried overnight before use.

2.3.5 Haematoxylin and Eosin (H&E) Staining

The composition of the components involved in haematoxylin and eosin staining will be described here. The theory behind this staining regime and the protocol will be described in Chapter 5 (Section 5.2). These stains were routinely prepared by Sheila Mcpherson in the Histology department.

Harris's haematoxylin is prepared by dissolving 2.5g of haematoxylin in absolute alcohol which is added to alum which has previously been dissolved in 500ml warm distilled water (Bancroft and Stevens, 1996). This mixture is boiled and either mercuric oxide (1.25g) or sodium iodate (0.5g) is carefully added. The stain is then rapidly cooled by plunging the flask into a sink containing cold water and chipped ice. Once cold 20mls of glacial acetic acid is added. Eosin Y is prepared by preparing a 1% solution with distilled water and 0.5ml of acetic acid is added to 1L (Bancroft and Stevens, 1996).

2.4 Immunocytochemistry

Immunocytochemistry is a method used to localise a specific protein within a cell using a specific antibody raised to the protein in question. This is a useful technique as it allows a protein to be localised to a specific cell type and often a specific cellular location. The procedure involves building up a sandwich of antibodies to increase the available binding sites for the avidin/biotin based linking system. This amplifies the signal obtained on addition of the horse-radish peroxidase based detection system

2.4.1 Immunoctyochemisty Protocol for Paraffin Sections

2.4.1.1 Procedure for detection with Diaminobenzidene (DAB)

Slide mounted tissue sections were deparaffinised in Histolclear (National Diagnostics, Fleet Business Park, Hull, UK) for 5-10 min and rehydrated by washes in decreasing concentrations of ethanol. The slides were then washed for 30 min in 270ml methanol containing 30ml of 30% hydrogen peroxide. The slides were washed twice in tris buffered saline (TBS; pH 7.4) for 5 min prior to the addition of a serum blocking solution which was diluted 1:5 in TBS and incubated for 30 min at room temperature (i.e. normal swine serum; Scottish Antibody Production Unit, Carluke, Scotland; SAPU). The primary antibody specific for the protein of interest was then applied at the working dilution (e.g. Aquaporin-1 used at 1:500 dilution). The antibody was diluted with the serum based blocking solution. The negative control slides were either incubated with pre-immune serum or control serum specific to the species the antibody was raised in (i.e. a polyclonal rabbit antibody negative control would be normal rabbit serum of an equal protein concentration). The tissue was either incubated overnight at 4°C or left at room temperature for 2 h.

Prior to the addition of the secondary antibody the slides were washed twice with TBS to remove any unbound antibody. The secondary antibody was applied to link to the primary and therefore had to be raised against the species used to raise the primary antibody (i.e. a rabbit polyclonal primary must be linked with a linking antibody raised against rabbit IgG). Therefore swine-anti-rabbit biotinylated (SARB; DAKO Ltd., Cambridge, UK) was incubated for 30 min at room temperature. After a further two washes in TBS an avidin-biotin/ hrp linking system (DAKO) was applied for 30 min. The avidin-biotin complexes bind to the biotinylated sites on the secondary antibody. After two final rinses with TBS the diaminobenzidene substrate was added (10ml TBS, 3.3µl 30% H_2O_2, 5mg DAB). This reacts with the horse-radish peroxidase to induce a brown precipitate at the site of antibody localisation. The reaction was stopped by immersing the slides in distilled water. The slides were moderately counterstained with heamatoxylin, dehydrated and cleared in xylene before being mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, UK) and coverslipped.

2.4.2 Immunfluorescence Protocol for Frozen Sections

Tissue sections were removed from -20°C storage and brought to room temperature. The sections were drawn around with a diamond pencil and then encircled in a ring of wax to ensure the slides did not dry out. Any excess OTC outwith this ring was scraped away with a disposable razor. The slides were washed twice for 5min in PBS to dissolve the OTC and non-specific binding sites were blocked by incubating the tissue for 20 min in 1% BSA/PBS solution. The primary antibodies were applied to separate tissue sections and left in a moist chamber at room temperature for 2 h. The slides were rinsed twice for 5 min in PBS before the addition of the secondary antibody which was linked to either FITC or CY3. The slides were incubated for a further hour. The slides were then rinsed in PBS 2x5mins before being mounted using Vextasheild H-100 mounting medium (Vector Labs, Burlington, California). Control experiments with no primary antibody were routinely performed and always gave negative staining.

2.5 Image Analysis

2.5.1 Digital Photomicroscopy (Paraffin Sections)

Tissue sections were examined using an Olympus Provis microscope (Olympus Optical, London, UK) and images were captured using a digital Kodak DCS420 camera (Eastman Kodak, Rochester, NY). Captured images were stored on a 8100 PowerPC computer (Apple MacIntosh, Apple Computer, Cupertino, CA) and images were compiled using Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA) before being printed using a Kodak XLS 8600 PS printer (Eastman Kodak).

2.5.2 Fluorescent Photomicroscopy (Frozen Sections)

The slides were examined using a Nikon FXA fluorescence microscope (Nikon, Surrey, UK). Profiles of tubules within the epididymis were captured using a digital Optronix 3-bit colour CCD camera and IP Lab Spectrum Software (Scanalytics, Vianna, Va.) running on a Power Macintosh 8500. Images were compiled using Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA) and printed using a Kodak XLS 8600 PS printer (Eastman Kodak, Rochester, NY).

2.5.3 Traditional Film Photomicroscopy

Black and white photomicrographs (Tmax 400 film) were taken of the immunofluorescent frozen sections to allow measurement and analysis. Images were shot using a Nikon FXA fluorescence microscope fitted with a Kodak film camera.

2.5.3.1 Development and Printing of Black and White Film

The Tmax film was removed from the canister in a dark room with no illumination and rolled onto a spool and placed in a light proof box. Tmax developer (Eastman Kodak Company) was diluted 1:5 with water and incubated with the film. The exact time depending on the temperature of the water. The developer was removed and the film washed under running water for 1 min. Any further developing was stopped by immersing in stop bath (16ml/L; Indicator Stop Bath, Eastman Kodak). After rinsing under running water for 1 min rapid fixer (Kodak) was added for 6 min. The fixer was prepared by adding 946ml of solution A to 1.9L warm water (27°C) and agitated before adding 104ml of Solution B and then add 850ml of water and left to cool. The film was then left under running water for 3-5 min before the addition of perm wash (47ml in 2L water; BKA Brandess-KAIC-AETNA Group, Inc,IL 60001). The film was washed for 1 min in distilled water and left to dry. The photomicrographs were then printed using images projected on to a 45 MTX Besseler enlarger. The exposure time was determined and the images printed using an automated Radioprint DD3700 Agfa Gavaert.

2.6 Protein Extraction from Efferent Ducts

Protein was extracted from adult rat kidneys and efferent ducts and from postnatal animals at day 18 for both control and DES (10μg) treated rats. Protein extraction was carried out on the pooled efferent ducts from 5 adult males. Samples were shredded with a hand-held homogeniser in 300μL chilled PBS containing 50μL protease inhibitor cocktail (Complete protease inhibitor cocktail, Boehringer Mannheim, East Sussex, UK). Protein extraction for the day 18 animals was as described above except that, efferent ducts were pooled from 6 treated and 6 control animals and the homogenisation volume was halved. The efferent ducts

are embedded in adipose tissue, so to remove this and any tissue debris, the samples were spun in a centrifuge at 3000 x g for 10 min at 4°C. The supernatant from below the fat layer was decanted and stored at -20°C.

2.6.1 Estimation of Protein Concentration

Due to the limited volumes of protein obtained from the efferent duct extractions a microtitre plate protein assay was optimised to determine the protein concentrations of samples. A standard curve of BSA was prepared by serial dilution ranging from 2mg/ml to 0.0156mg/ml. The total volume in each microtitre well was 150 µL, comprising 25µL of sample and 125µL of BioRad protein assay reagent (Bio-Rad Laboratories, Hemel Hemstead, UK). This reagent detects the concentration of protein based on a dye colour change in response to protein binding (Bradford, 1976). The reagent was diluted 1:5 with deionised distilled water and filtered. Each of the unknown protein samples were added in a sample volume of 25 µL and at least two different dilutions (i.e. 1:10 and 1:50). After the addition of the BioRad reagent the plate was swirled gently and after 5-60 min the plate was read in a Labsystems Multiskan® Mcc/340 plate reader and the optical density measured at 620nm. A standard curve was drawn and the protein concentrations of the samples manually determined from the graph.

2.7 One Dimensional Gel SDS-PAGE

One dimensional gel SDS-PAGE was performed based on the technique described by Laemmli (1970) (Laemmli, 1970). A Protean electrophoresis system with a 300xi power unit (Bio-Rad Laboratories) was used to perform the electrophoresis. Prior to and during use, the apparatus was cooled to 10°C using a chiller/ heater circulator unit (Betta-Tech Controls, Newport Pagnell, Bucks, UK). Protein samples were separated on 12% w/v acrylamide gels. Each gel was composed of a total of 32ml (14ml 30% acrylamide, 8ml 1.5M Tris-HCl (pH 8.8) and 9.6 ml distilled water). Gels were degassed and polyimerised by the addition of 300µl 1% ammonium persulphate and 9µl TEMED (Sigma). Once cast, the gel was overlain with water-saturated-isobutanol (Aldrich Chemical Co.) until set. This was removed and the gel washed with distilled water. The gel was then ready for use or overlain with 0.375M Tris-HCl and stored overnight at 4°C. After storage the gel was left to come to room temperature while the stacking gel was prepared (consisting of 1.3 ml 30% acrylamide, 2.5 ml 0.5M Tris-HCl, 6.2 ml double distilled water) and polymerised by the addition of 10µl TEMED (Sigma) and 50µl 1% ammonium persulphate. A fifteen well comb was inserted into the gel and it was left to polymerise.

Prior to the addition of the protein samples they were mixed with an equal volume of SDS sample buffer containing 1.5% w/v Tris dissolved in double distilled water and HCl used to adjust the pH to 6.75. To this was added 4% w/v SDS, 2% w/v dithiothreitol (DTT) and 0.05% w/v bromophenol blue and the final volume made to 20ml with double distilled water. After the addition of the sample buffer the samples were boiled for 5mins and centrifuged for 2 min at 1000xg and loaded into the stacking gel. One lane was reserved for molecular weight markers (phosphorlase b, 97 kDa; albumin, 67 kDa; carbonic anhydrase, 43 kDa; trypsin inhibitor, 20.1 kDa; α-lactalbumin, 14.4 kDa; Pharmacia, Milton Keynes, UK). The gel was enclosed in a tank containing an electrolyte buffer (0.3% w/v Tris base, 1.44% w/v glycine and 0.15% w/v SDS (all from Sigma) in double distilled water. Electrophoresis was performed at 35mA until the samples had cleared the stacking gel and raised to 38mA for 3.5 h. After electrophoresis the gel was electroblotted for Western blot analysis.

2.8 Western Blot Analysis

This technique detects a specific protein resolved using SDS-PAGE. The technique uses the specificity of antibody/ligand interactions to bind to the protein of interest. As SDS breaks disulphide bonds and induces alterations in the tertiary structure of the protein, antibodies raised to conformational epitopes may not be successful in this method. Two detection methods were employed as the first (NBT detection) was not successful in producing a specific band.

2.8.1 Nitroblue Tetrozolium (NBT) detection

Protein samples which were separated using 1-D SDS-PAGE were electroblotted by semi-dry transfer (Novablot apparatus; LKB Pharmacia, Uppsala, Sweden) onto a PVDF membrane (Immobilon-P Millipore, Watford, UK) based on the technique described by Towbin *et al.*, (1979) (Towbin *et al.*, 1979). The transfer buffer consisted of 0.06% w/v Tris base, 0.288% w/v glycine, 0.02% w/v SDS and 20% w/v methanol (BDH, Merk Ltd., Leicestershire, UK). The gel was blotted for 90 min at a current which was calculated by determining the area of the gel and multiplying it by the constant 0.8. This generally worked out at between 160-180 mA. After blotting onto nitrocellulose, the lane containing the molecular weight markers was removed and stained with coomassie blue (0.1% w/v coomassie blue in 50% methanol and 7.5% w/v acetic acid) for at least 30 min. The strip was destained (50% methanol:5% acetic acid v/v) until the staining of the MW markers was optimal.

The remainder of the membrane was blocked for 2 h at room temperature in TBS-Tween (Tris-buffered saline pH 7.4, containing 50mM Tris-HCl, 150mM NaCl and 0.05% v/v Tween 20) containing 5% normal sheep serum (SAPU). The membranes were incubated

overnight at 4°C with the primary polyclonal antibody to Aquaporin-1 which was diluted 1:5000 with blocking buffer. The control blot was incubated with the same dilution of pre-immune serum. The blots were rinsed 3 times for 10 min in TBS-Tween prior to the addition of the secondary antibody. Sheep-anti-rabbit IgG (SAR; Serotec, Oxford, London) was diluted 1:500 in TBS and incubated for 45 min at room temperature. A further three 10min washes with TBS-Tween were performed before the addition of 1:2000 dilution (in TBS) of rabbit alkaline phosphatase anti-alkaline phosphatase conjugate (Serotec) for 45 min. The blots were given two final washes in TBS-Tween, one in TBS and a further 2 min rinse in equilibrating buffer (100mM Tris, pH 9.5, 100mM NaCl and 50mM $MgCl_2$). The membranes were developed immediately in 25ml equilibrating buffer to which 0.3mg/ml nitroblue tetrozolium (NBT), 0.175mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 0.24mg/ml levamisole (all from Sigma) were added. Developing was performed in the dark and the reaction was stopped by immersing the blot in distilled water. This detection method gave a large number of non-specific bands which masked any positive identification of the Aquaporin-1 protein so a more sensitive detection method was employed as described below.

2.8.2 ECL Detection

The 1-D gel was electroblotted as described in Section 2.8.1. The blots were then left to block overnight at 4°C in 5% normal swine serum (NSS; SAPU) in TBS-Tween. The primary antibody (rabbit polyclonal raised to aquaporin-1; diluted to 1:5000 in TBS) was incubated at room temperature for 2h and the control blot was incubated in pre-immune serum (1:5000). The MW marker strips were removed and washed in NSS-TBS-Tween. Membranes were then washed for 40 min in large volumes of TBS-Tween with several changes of buffer prior to the addition of the secondary antibody. Blots were incubated for 1 hour in swine-anti-rabbit peroxidase (1:5000 in TBS) containing 5% NSS and then washed for a further 40 min in TBS-Tween. The MW markers were incubated with streptavidin peroxidase(1:3000 in TBS-NSS) for 1 h. The blots were then ready to be developed in a dark room. ECL (Amersham Life Science, Buckinghamshire, UK) detection was performed as per the manufacturers guidelines Hyperfilm (Amersham) was placed over the blots for the desired time and the film was developed and fixed. A specific band appeared at 29 KDa signalling the detection of AQP-1.

2.9 Ribonucleic Acid (RNA) Extraction

RNA extraction was performed using a commercially available product, Tri-Reagent™ (Sigma) which follows the method described by Chomczynski, (1993) (Chomczynski, 1993).

Tissues were dissected from rats (testis, caput, corpus, cauda epididymis, prostate, kidney, adrenals, heart, lung, liver, intestine and the efferent ducts which were carefully dissected free from surrounding adipose tissue) and pieces were placed into 10-15ml of chilled Tri-Reagent and homogenised on ice until the tissue was completely dissociated and then left on ice. Prior to the addition of 4 ml chloroform (Sigma) the mixture was left at room temperature for at least 5 min. Vigorous mixing on the addition of chloroform induced the formation of an emulsion. After standing at room temperature for 15 min the mixture was centrifuged at 10,000 x g at 4°C for 20 min. The upper aqueous phase was decanted to a new vial and an equal volume of isopropanol (stored at -20°C) was added. This induced the precipitation of RNA which was left to stand at room temperature for 10 min and centrifuged as above. The resulting RNA pellet was washed with 75% ethanol, air dried then dissolved in water and stored at -70°C. The purity and concentration of the RNA sample was determined by reading the optical density (OD) at 260 and 280nm. The ratio for each sample was calculated to give an estimation of the purity of the RNA, a ratio of 2.0 was taken to be pure. The concentration of the sample was calculated from the 260nm reading. An OD of 1.0 is equivalent to an RNA concentration of 40μg RNA/ml.

2.10 Oligonucleotide Primers

Oligonucleotide primers for CFTR were chosen by Dr Philippa Saunders and sent to Genosys (Genosys Biotechnologies (Europe) Ltd., Cambs, UK) for synthesis. Primers were chosen using the following guidelines. Primers should ideally have a 50% G+C content and have an annealing temperature within the range 56-62°C. The primer and the expected PCR product should be within the coding regions but avoiding conserved regions. Ideally primers should be picked on different exons so that the RNA-specific PCR product is a different size from any bands arising through contaminating DNA. The length of the primer determines both the specificity and the annealing temperature. A primer of 18-24 nucleotide bases is sequence specific as long as the annealing temperature is set within a few degrees of the dissociation temperature for the template/primer duplex. Using primers of minimal length (18 bases) ensures a melting temperature of around 54°C or higher and a high probability of sequence specificity. The 3' end of the primer is critical for successful PCR. If a conserved amino acid can be used and serve as the first three bases in the primer, it allows a perfect base pairing between the template and the 3' end. All primer sequences were checked through GenBank to ensure that they did not match to any other known sequences.

2.10.1 Concentration of Primers

The concentration of primer required for a 100μl PCR reaction is 0.05μM. To calculate the volume of primer required the OD at 260nm was checked by adding 4μl primer to 796μl

distilled water. The OD was multiplied by 4000. The molecular weight of the primer was calculated and divided by 20. This value was divided by the value obtained by multiplying OD by 4000 to give the volume (µl) of primer required to give 0.05µM in 100µl. Prior to the use of specific primers, a cDNA pool from the tissue of interest must be synthesised to provide a template to which the specific primers can bind.

2.11 cDNA Library Synthesis

This procedure was performed using reagents from Boehringer Mannheim. cDNA was synthesised using random hexanucleotides, which bind to RNA at any complementary site then performing RT-PCR to amplify pools of cDNA. RNA (2.5µg) from a variety of tissue were used as a template for DNA synthesis. This was incubated together with 2µl of random primer and added sterile water to bring the total volume to 9µl. This mixture was heated at 70°C for 10 min and centrifuged briefly before the addition of 4µl reverse transcriptase buffer, 2µl 0.1M DTT and, 1µl each of 10mM dATP, dCTP, dGTP, dTTP. This was heated to 42°C for two mins before 1µl of reverse transcriptase was added and mixed by pipetting. This was incubated at 25°C for 10min followed by 50 mins at 42°C. The reaction was stopped by adding 4µl 50mM EDTA. 2µl of this cDNA pool was used as a template for PCR with specific primers.

2.12 PCR Amplification Using Specific Primers

The cDNA library can be probed with specific primers to determine whether a specific RNA message is present within the tissue from which the cDNA pool was synthesised. Thermal cycling, using the polymerase chain reaction (PCR), allows the rapid synthesis of millions of copies of a specific section of DNA. RNA polymerase (Taq Polymerase; Perkin Elmer, Ltd., Cheshire, UK) catalyses the synthesis of the DNA strand which lies between the 3' and 5'primer pair, which will be of known size. A supply of reaction buffer, dNTPs, and the optimised concentration of magnesium chloride are required for successful PCR. The PCR mixture is overlain with mineral oil to prevent evaporation and placed in a thermal cycler for 25-35 cycles.

To check that the size of the PCR product was correct 10µl was run out on a 1.5% agarose gel (75ml mini-gel contains; 1.13g agarose (Boehringer Mannheim), 75ml 1x TBE buffer (0.089M Tris base, 0.089M boric acid and 10mM EDTA)). The agarose was melted and 2µl of ethidium bromide added (Sigma) to allow UV visualisation of the PCR bands. The PCR product was mixed with 5µl of OJ ('orange juice'; 0.25% orange G (Sigma), 15% w/v ficoll and 0.5M EDTA at pH 7.0). The samples were electrophoresed by running in 1x TBE at 100V for 1 h alongside pGEM DNA markers (range 36-2645 base pairs, Promega). The gel

was viewed under UV light and photographed. A single band of the correct size should be visible in each sample.

2.13 Cloning of PCR Product into Plasmid Vector

The cloning of PCR products synthesised using Taq polymerase can be performed using the TOPA TA Cloning® Kit Dual Promoter (Invitrogen BV, Holland). The cloning reaction was performed by adding 2µl of PCR product to 1µl of pCR-topo vector and 2µl sterile water. This was mixed gently and incubated for 5 min at room temperature then briefly centrifuged and placed on ice. The vector containing the PCR product was introduced into competent cells; this kit uses One Shot™ competent *E. coli* . The transformation reaction was performed by adding 2µl of 0.5M β-mercaptoethanol to a vial of competent cells and mixed by stirring gently with a pipette tip prior to the addition of 2µl of the cloning reaction. This was incubated for 30min before the cells are heat shocked at 42°C for 30 sec. The cells were then cooled on ice for 2mins and 250µl of SOC medium added and mixed. The tube was left in a shaker at 37°C for at least 30 mins and then placed on ice. The *E.coli* were then spread on agar plates containing ampicillin (Penbritin Injection, Beecham Research) which had been previously spread with 40µl of 40mg/ml X-gal and 40µl of 100mM IPTG. The plates were left overnight at 37°C.

2.13.1 Analysis of Positive Clones - Direct Lysis PCR

This technique is a quick way to check that the transformed cells have an insert of the correct size. Only white colonies should be analysed. A white colony was placed in 50µl plasmid lysis buffer and heated at 95°C for 5min. After being centrifuged at 13,000 rpm for 5min, 5µl of the supernatant was used as a template for PCR using the vector promoter sites T7 and SP6 as template primers. The size of the insert was checked by running the PCR product on a 1.5% agarose gel as described in section 2.12.

2.13.2 Plasmid Preparation and Glycerol Stocks

2.13.2.1 Glycerol Stocks

Plasmid stocks were prepared by picking a colony off the agar plate and growing it overnight at 37°C in 10ml LB (Luria Bertani) broth containing 50µg/ml ampicillin. Prior to plasmid preparation, 700 µl of bacterial stock was added to 300µl of 50% glycerol and frozen to provide a future stock of *E.coli* from which fresh plasmid could be grown.

2.13.2.2 Plasmid Preparation

Plasmid DNA was isolated from remainder of the culture using the alkaline lysis method in Wizrad™ DNA purification system (Promega). The culture is centrifuged for 15 min at 1,600

x g and the pellet was re-suspended in 'Resuspension solution' (50mM Tris-HCl pH 7.5, 10mM EDTA and 100µg/ml RNase). The tubes were inverted after addition of 300µl of the cell lysis solution (0.2M NaOH and 1% SDS) and the solution neutralised in 300µl of neutralisation buffer (1.32M potassium acetate and 6.4% glacial acetic acid (pH 4.8). The mixture was centrifuged for 5min at 12,000 x g resulting in the sedimentation of bacterial genomic DNA. One ml of DNA purification resin was then applied to the plasmid suspension before it was passed through a miniprep column. The column only retards plasmid DNA. The column was washed (200mM NaCl, 20mM Tris/HCl, pH 7.5, 5mM EDTA and 47.5% v/v ethanol) and the plasmid eluted from the column by the addition of 100µl of hot (~70°C) water.

2.13.3 Plasmid DNA Purity and Concentration

The purity of plasmid DNA was determined by running 1µl of plasmid on a 0.8% agarose gel. Samples were separated in parallel with pGem DNA markers. Under UV light, pure plasmid DNA appears as two bands, one for circular DNA and the other representing supercoiled DNA. Supercoiled DNA is more compact and therefore runs more rapidly through the gel. The approximate concentration was determined by running a plasmid sample of known concentration (200ng; pBR322, Promega) on the gel and comparing the intensity of the band.

2.14 *In situ* hybridisation

Two different detection methods were employed to try and localise specific RNA species to the cells of a specific tissue. Abundant mRNAs can generally be detected without the use of radioactivity. This method was employed unsuccessfully for the detection of CFTR and so radioactive detection was also attempted.

2.14.1 Non-Radioactive In Situ Hybridisation

2.14.1.1 Probe Preparation

Plasmid DNA containing a specific insert for CFTR was prepared as described in sections 2.10- 2.12. The plasmid was linearised (1µg DNA, reaction buffer (10-50mM Tris-HCl, 5-10mM $MgCl_2$, 50-100 mM NaCl and 1mM dithiothreitol; Boeringher Mannheim), 10U restriction enzyme (Boerhinger Mannheim) and pure water was added to make up to a volume of 40µl followed by incubation for 1-2 h at 37°C. The restriction enzyme used depends on the plasmid vector being digested and the direction of synthesis of the riboprobe. To check whether the enzyme had cut the plasmid properly, 3µl of the reaction was run on a 0.8% agarose gel alongside an uncut sample of plasmid. Digested DNA was extracted with Tris-buffered phenol:chloroform:isoamylalcohol 25:24:1 v/v (pH 8.0), and then precipitated with

1/10th volume 3M sodium acetate (pH 5.5) and 2.5 volumes absolute ethanol overnight at -20°C. The linearised DNA was pelleted, dried and resuspended in 10µl pure water.

2.14.1.2 Preparation of Digoxigenin Labelled Riboprobes

Riboprobe synthesis was performed using 1µg of linearised plasmid DNA. This was added to the following reaction: 10mM DTT (Promega), 1mM each of rATP, rCTP, rGTP (Boehringer Mannheim), transcription buffer (1x transcription buffer containing 40mM Tris-HCl (pH 7.9), 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl and 0.35mM Dig-labelled UTP (Boehringer Mannheim) with 20U RNase inhibitor (Promega) and the appropriate RNA polymerase (either T3 or SP6; Boehringer Mannheim). This was incubated for approximately 2 h at 37°C prior to running out 2µl of the reaction on a 0.8% agarose gel to detect the presence of two bands corresponding to 28 and 18 S RNA. Enzymes and salts were removed by two rounds of phenol/chloroform extraction. The RNA was precipitated with sodium acetate and ethanol overnight at -70°C and recovered by centrifugation. The RNA was suspended in 50µl sterile water and further purified by passage through an RNA spin column (Nu-clean R50, IBI).

2.14.1.3 Pre-treatment and Hybridisation of Tissue

Slide mounted paraffin tissue sections were cleared in Histoclear for 10 min and rehydrated by passing through an alcohol series of decreasing concentration. Tissue was placed in 0.2M HCl for 20 min and then washed twice with distilled water. The tissue was incubated in 2µg/ml Proteinase K (Sigma) in buffer (20mM Tris-HCl pH 7.4, and 50mM EDTA) at 37°C for 40 min to remove excess protein from the tissue section which aids the hybridisation of the riboprobe. Proteinase K was inactivated by incubating the slides at 4°C in 0.2% glycine for 20 min and then washed in 0.1M triethanolamine TEA pH 8.0 (Sigma). The slides were acetylated to reduce any ionic charges produced by protein digestion by the addition of 0.25% acetic anhydride (Sigma) in TEA buffer. The slides were given a final wash in 6x STE (1x STE contains 150mM NaCl, 2.5mM Tris and 0.25mM EDTA) before being incubated for 2-4 h in prehybridisation buffer containing 6x STE, 1x Denhardt's solution (50x Denhardt's: 5g BSA, 5g polyvinylpyrolidine and 5g ficoll in 500ml solution), 125µg salmon sperm DNA (Sigma), 125µg/ml yeast transfer RNA (Sigma) and 50% deionised formamide for 2-4 h in a humidified chamber at 35°C. The probe was added to the hybridisation buffer which had the same composition as prehybridisation buffer except for the addition of 10% v/v dextran sulphate. The probe was added at a concentration of 2µl with 40µl of buffer being allowed for each slide which was then coverslipped with Gel Bond film (Flowgen Instraments Ltd.). This was hybridised overnight at 35°C in a humidified chamber.

The slides were washed twice for 5 min in 4x SSC to wash off any unhybridised probe before being incubated in RNase A at a concentration of 20μg/ml in buffer for 30 min at 37°C. Slides were rinsed in 2x SSC (5 min) at room temperature followed by 30 min incubation in 0.1x SSC/30% v/v formamide at 30-40°C.

2.14.1.4 *In Situ* Hybridisation - Development of Signal

Slides were washed in TBS (2x 5 min washes) before they were blocked with normal sheep serum (1:5 dilution with TBS) for 30 min. The slides were rinsed with TBS prior to the addition of the linking antibody (anti-DIG conjugated to alkaline phosphatase Fab fragments raised in sheep; Boehringer Mannheim) used at 1:300 in TBS and incubated for 2 h. Excess antibody was rinsed with TBS washes (2x 15 min). The development was performed as described in section 2.8.1. The sections were incubated with this solution in the dark at room temperature until staining was optimal (generally overnight) prior to being washed in water. The slides were then dehydrated and coverslipped.

2.14.2 Radioactive in situ Hybridisation

2.14.2.1 Preparation ^{35}S-Labelled Riboprobes

Plasmids were cut as described in Section 2.13.1.1. The riboprobe was synthesized by the addition of 3.5μl sterile water, 5μl of 5x transcription buffer, 1μl of RNasin, 1μl 100mMDTT and 1μl of each of the following 10mM rATP, rCTP and rGTP and 0.5μl of 100mM UTP. Together with 5μl of the linearised plasmid , 5μl ^{35}S UTP and 1μl of RNA polymerase SP6 or T7, the sample was incubated at 37°C for 1 h and a further 1μl of RNA polymerase was added. After a further hour 1μl Dnase was added and the solution was passed down a DEPC-100 column (Chroma Spin-100) to remove unincorporated nucleotides. The radioactivity level was determined by adding 1μl of the probe to 4ml scintillation fluid and reading it in a β-counter. The volume of probe required to coat each slide with 10^6cpm probe was then determined.

2.14.2.2 Pretreatment and Hybridisation of Tissue

This was performed as described in Section 2.13.1.3.

2.14.2.3 Development of *In Situ* Hybridisation

Once the slides were given their final wash they were dehydrated in graded ethanols containing ammonium acetate and left for an hour to air dry. The slides were then dipped in emulsion (NBT-2 autoradiography emulsion, Kodak) at 45°C and placed in a light- proof humid box and left for several hours at room temperature prior to the addition of a desiccant. The slides were exposed for two weeks at 4°C before being developed. Slides were developed

by being incubated in filtered developer (Kodak D-19 developer diluted into 5L, Kodak), and then cooled to 14°C for 4 mins. Before being fixed in Kodak GBX fixer and replenished for 10 min, the slides were washed in distilled water. Once developed the slides were washed in water and then counterstained as described in Section 2.4.1.1.

Chapter 3 Oestrogen Receptor-Beta Immunoexpression in the Male Marmoset Reproductive Tract

3.1 Introduction

The human oestrogen receptor (ER) was cloned in 1986 and was classed as a member of the steroid receptor gene superfamily which includes the other sex steroid receptors along with the thyroid hormone receptor, glucocorticoid receptor and retinoic acid receptor (Green *et al.*, 1986). The contradictory evidence in the literature concerning the sites of expression of the oestrogen receptor (reviewed in section 1.7.1) should perhaps have alerted scientists to the possibility that more than one form of oestrogen binding protein existed. However, it was not until 1996 that a second isoform of ER was detected and cloned from rat prostate and ovary (Kuiper *et al.*, 1996). This second form was named oestrogen receptor-beta (ERβ) and the older isoform was renamed ER-alpha (ERα). The isolation of this second isoform has opened a new field in scientific research and has broadened the understanding of both the possible roles of oestrogen and the molecular dynamics of oestrogen receptor activation. The work in this chapter aimed to determine the sites of immunoexpression of ERβ within the male marmoset testis and epididymis and to compare these with the expression of ERα and the androgen receptor (AR). This introduction summarises what has been discovered about ERβ over the past 3 years since its identification.

ERβ has currently been cloned in the rat (Kuiper *et al.*, 1996), mouse (Tremblay *et al.*, 1997), human (Enmark *et al.*, 1997) and sheep (Walther *et al.*, 1999). The human gene has been mapped to chromosome 14q22-24 while the murine gene resides in the central region of chromosome 12 (Enmark *et al.*, 1997), (Tremblay *et al.*, 1997). Sequence comparisons have determined that ERβ is highly homologous to ERα (Kuiper *et al.*, 1996), and has a similar arrangement of the functional domains characteristic to members of the steroid receptor superfamily. The N-terminal region contains the transactivation domain which can interact with transcriptional components, and this region shares the least homology with ERα. The DNA binding domain is highly conserved (there is 95-97% homology between the two isoforms) and contains two zinc fingers which are required for inducing receptor dimerization and DNA binding (Kuiper *et al.*, 1996). Finally, the C-terminus contains the ligand binding domain which is essential for nuclear translocation, ligand-dependent transactivation, receptor dimerization and interactions with co-repressors and activators (Chang and Prins, 1999).

Within the DNA binding domain there are two domains (P-box and D-box which bind to oestrogen response elements (ERE) of oestrogen responsive genes) that are identical between ERα and β; thus predicting that ERβ will bind to the same EREs that have already been

identified for ERα (Kuiper *et al.*, 1996). The amino acid sequence of the ligand binding domain of ERβ shares only 55-60% homology with ERα (Kuiper *et al.*, 1996), (Mosselman *et al.*, 1996), (Tremblay *et al.*, 1997). Variation within the ligand binding domain may suggest that the two isoforms of ER have different affinities for ligand.

Ligand binding affinities to various oestrogen metabolites and oestrogenic compounds have been assessed using recombinant ERα or ERβ. Both receptor isoforms bound 17β-oestradiol with high affinity but the dissociation constant for ERβ was generally half that of ERα (Kuiper *et al.*, 1996), (Kuiper *et al.*, 1997), (Tremblay *et al.*, 1997). ERβ has been shown to bind many xenoestrogens (polychlorinated biphenols (PCBs), dichlorodiphenyl-trichloroethane (DDT) and metabolites, alkylphenols and Bisphenol A) with an affinity that is generally 1000 times lower than that for 17β-oestradiol (Kuipier *et al.*, 1998). However, phytoestrogens (genistein, coumestrol and zearlenone) were found to have a 10-fold higher affinity for ERβ than ERα (Kuiper *et al.*, 1997; Kuipier *et al.*, 1998) as did Bisphenol-A and methoxychlor (Kuiper *et al.*, 1997). The xenoestrogens used in this assay were able to induce reporter gene expression at a consensus ERE using a human ERβ expression plasmid (Kuipier *et al.*, 1998). This may suggest that tissues with a high ERβ or combined ERα and ERβ expression may be more vulnerable to potential effects of environmental chemicals (Chang and Prins, 1999).

After ligand binding, steroid receptor proteins link to another steroid receptor forming a dimer which then translocates to the nucleus. It is known that some steroid receptors are capable of dimerizing with other members of the steroid family to form heterodimers. Experiments have been performed to determine whether ERβ forms homodimers or is capable of interacting with ERα to form heterodimers. Studies using human and mouse ERβ have shown that ERβ is capable of forming heterodimers with ERα in vitro and in COS-7 cells (Pettersson *et al.*, 1997), (Ogawa *et al.*, 1998). The formation of heterodimers suggests the potential for three oestrogen-mediated signals in cells (ERα/ERα, ERβ/ERβ and ERα/ERβ) which express both isoforms of ER which may therefore induce the transcription of three unique sets of genes.

The cloning of ERβ has led to the publication of a number of papers suggesting the existence of multiple forms of this protein. Petersen and co-authors isolated four variants (ERβ 1-4; with ERβ1 being the originally cloned receptor). ERβ2 had an in-frame insertion of 54 nucleotides which corresponded to an additional 18 amino acids being inserted into the ligand binding domain (Petersen *et al.*, 1998), (Hanstein *et al.*, 1999). ERβ2 was expressed at similar levels to ER-β1 in the ovary, prostate, pituitary and muscle but ER-β2 had a lower affinity for oestradiol than did either ERα or β1 (Petersen *et al.*, 1998). Two further variants (ERβ4,5)

were identical to ER-β1 and ER-β2 respectively except that they had a deletion of 117bp. These bases encompassed the coding region of the second zinc finger of the DNA binding domain. All 4 variants were translated into functional proteins (Petersen *et al.*, 1998). Another study isolated 5 isoforms of ERβ from a human cDNA testis library. All five isoforms were shown to diverge at a common position within the predicted helix 10 of the ligand binding domain, which the authors predict is consistent with different exon usage (Moore *et al.*, 1998).

There has also been some debate regarding the true transcriptional start site for the ERβ gene as one study found that the N-terminus contained an additional open reading frame, both in-frame and upstream of the published ERβ clones (Bhat *et al.*, 1998). This long form of the receptor is more active than the 'truncated' variant at activating ERE-based reporter genes, which might suggest that the extended N-terminus amino acids have a role in modulating oestrogen responsive gene expression, at least *in vitro* (Bhat *et al.*, 1998). An extended N-terminus was also reported by Ogawa and co-authors (1998), they suggested that an additional 53 amino acids were present in the extended N-terminus and that this variant was capable of forming heterodimers with ERα (Ogawa *et al.*, 1998).

ERβ expression has been determined in a number of tissues within the reproductive systems of both sexes, the brain, urinary system, cardiovascular system and bone using various methods from RT-PCR, in situ hybridisation, RNase protection assays, Northern blots and immunocytochemistry (see Chang and Prins, 1999). As bone growth was affected in humans with inactivating mutations of aromatase and ERα, the sites of ERβ expression in this tissue will be important for determining the complete role of oestrogen in bone physiology. Immunocytochemistry using two different antisera demonstrated ERβ staining in tissue samples collected from four female prepubertal patients. Staining was located in hypertrophic epiphyseal chondrocytes suggesting that ERβ (as well as ERα) plays a role in longitudinal bone growth (Nilsson *et al.*, 1999). When the experimental research of this thesis was started in 1996, the sites of expression of ER-β within the male reproductive tract were not known, aside from the bands detected in prostate and testis which were illustrated on the Northern blot published by Kuiper (1996). This chapter examines the immunolocalisation of ERβ within the testis and epididymis of the marmoset monkey from perinatal life until adulthood.

3.2 Experimental Procedures

3.2.1 Tissue collection

Marmoset tissues were collected with the testis and epididymis attached. Donor animals were classed as neonates, infants, prepubertal or adults (aged 1 day to 8 weeks, 18-24, 54-62, 92-112 weeks, respectively; n= 2-4 per group). Tissues were collected from control animals by

Dr Hamish Fraser and were from another experiment. The tissue was fixed in Bouins fluid and processed into paraffin blocks (as described in section 2.3.4).

3.2.2 Antibody Production

The antibody used in this study was synthesised under the direction of Dr Phillipa Saunders. A peptide raised to the hinge domain of ERβ (CAGKAKRSGGHAPRVREL) was synthesised by Affitini Reagents (Exeter, UK). This peptide was injected into sheep; immunisations and recovery of antisera were all carried out by Diagnostics Scotland, Carluke, Lanarkshire, UK. The serum was then affinity purified by Dr Saunders using the following protocol. The polyclonal antiserum was purified against the unconjugated form of the immunising peptide as follows: the antiserum was mixed with 2x volume of sodium acetate (60 mM; pH 4) and stirred vigorously during the addition of 0.75ml caprylic acid (Sigma, St Louis Missouri) per 10ml of antiserum. The resulting cloudy solution was centrifuged at 3000g for 30 min at 4°C, the supernatant was filtered through Whatman No. 1 paper and dialysed extensively against phosphate buffered saline (PBS; pH 7.4). The antibody was used at a dilution of 1:500 and antiserum pre-absorbed with the immunising peptide was used as a negative control.

3.2.3 Immunoctyochemistry on paraffin tissue sections

Immunocytochemistry was performed as described in Section 2.4.1 except an antigen retrieval step was introduced after the methanol/hydrogen peroxide wash. The slides were pressure cooked in glycine buffer (50mM; pH3.5 + 0.01% EDTA) for 5 min and left to stand for 20min. The heat treatment and the pH of the buffer used appear important in antigen retrieval. This protocol was optimised by Mr M Millar. The marmoset tissue was also blocked in 0.01% avidin in PBS for 30 min before being incubated in 0.001% biotin for 30 min. This step was performed to quench any endogenous biotin activity within the marmoset testis. The primary antibody was then added. The negative control was pre-absorbed antibody which was prepared 24h before use. The peptide used to immunise the sheep was added to the antibody at 10 times the protein concentration of the antibody. The protocol was then followed as described in Section 2.4.1. The secondary antibody was a rabbit anti-sheep biotinylated (Serotec).

3.2.4 Androgen Receptor Immunocytochemistry

Immunocytochemistry was performed using a commercial rabbit polyclonal antibody (Novocastra). The protocol used was as described in Section 2.4.1, except that an antigen retrieval process was performed after the methanol block. Slides were pressure cooked for 5 min in 0.01M citrate buffer (pH 6.0) and left to stand for 20 min. Instead of incubating the

primary antibody overnight, it was left at room temperature for 2 hours. The antibody was used at a dilution of 1:20.

3.2.5 Oestrogen Receptor α Immunocytochemistry

Immunocytochemistry was performed using a commercial monoclonal antibody (Novocastra) which had previously been validated (Fisher *et al.*, 1997). The slides were immunostained using NBT (nitroblue tetrazolium) which form a dark blue precipitate at sites of antibody localisation or DAB (diaminobenzidine) which forms a brown precipitate. The immunostaining procedure is essentially similar to that described in Chapter 2 (section 2.4.1) except that there was no methanol block. The slides underwent an antigen retrieval step as described in Section 3.2.4. The primary antibody was used at a dilution of 1:20 diluted in normal rabbit serum (NRS). The secondary linking antibody was mouse-anti-rabbit biotinylated used at 1:500 in NRS. After the final wash in TBS the detection process involved incubating the slides in 100mM Tris-MgCl buffer (100mM NaCl and 50mM MgCl; pH 9.5) before the addition of the NBT developing solution (337.5 μg/ml NBT, 175 μg/ml 5-bromo-4 chloro-3-indolylphosphate and 0.001% levamisole in 10ml of Tris-MgCl buffer). The slides were then counterstained and coverslipped.

3.2.6 Photomicroscopy

The slides were photographed as described in Section 2.5.1.

3.3 Results

3.3.1 Oestrogen Receptor β Immunolocalisation in the Testis and Epididymis of the Marmoset

Figure 3.1 illustrates the localisation of ERβ by immunocytochemistry within the marmoset from day 1 to peripubertal life at around 62 weeks of age. Panels (a) and (b) respectively illustrate ERβ staining within marmoset bladder epithelium (positive control tissue) and the negative control slide treated with antibody preabsorbed with cognate peptide. Bladder was shown to be ERβ positive by Saunders *et al.*, 1998. Figure 3.1(c) illustrates ERβ staining of blood vessel endothelial cells and the surrounding adipocytes located within the epididymal fat pad. Similar staining was observed in endothelia and adipocytes throughout the body. This image was taken from tissue obtained from an 8 week old marmoset but similar staining was evident from day 1 to adulthood. Panels d-f are from a day one postnatal marmoset and depict a low power image of the testis and overlying epididymis, and higher power images of the seminiferous cords and developing epididymis respectively. The trend from panel (d) suggests that ERβ is most evident within the developing epithelial tissues of the testis and epididymis, although there was obvious stromal cell staining within the epididymis and at the

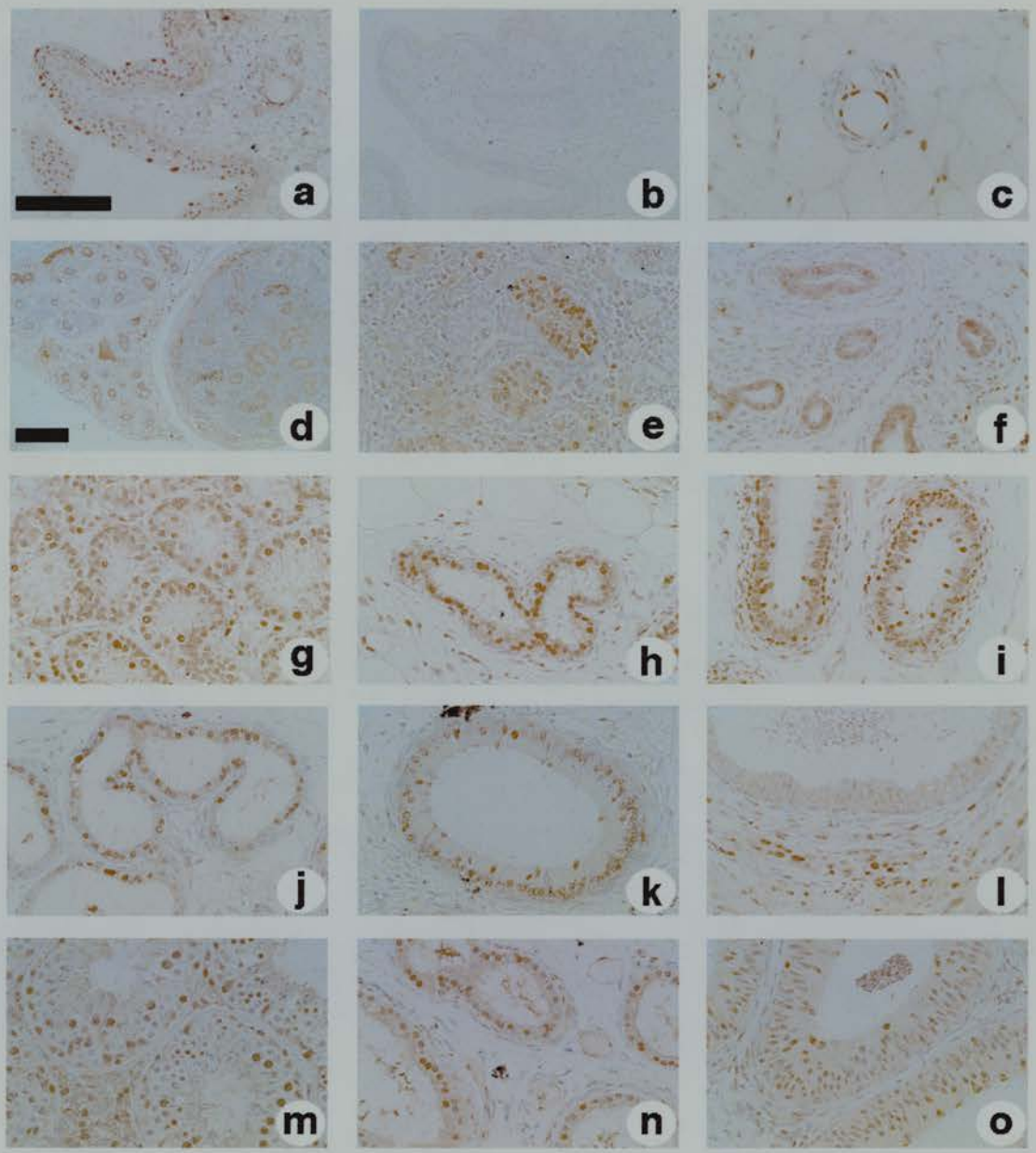

Figure 3.1 Immunocytochemical Localisation of ERβ Within the Marmoset from Perinatal Life to Adulthood

Panel (a,b) represent the positive control tissue, the bladder with ERβ localisation and staining pattern observed after the antibody is preabsorbed with peptide. Panel (c) shows endothelial cells from a blood capillary and surrounding adipocytes. Panels (d-f) illustrate ERβ immunolocalisation of day 1 postnatal marmoset (d) testis (e) and epididymis (f). An infant marmoset (8 weeks) is depicted in panels (g-i), testis (g), efferent ducts (h) and caput epididymis (i). Peripubertal animals (62 weeks) illustrated in panels (j-l) representing efferent ducts (j), caput epdidymis (k) and vas deferens (l). The adult marmoset ERβ is depicted in panels (m-o), testis (m), efferent ducts (n) and caput epididymis (o). All images are x400, except (d) x100. The scale bar in (a) denotes 100μm and that in (d) denotes 200μm.

circumference of the testis within the apical layer of cells. There were many developing blood vessels in this region. It is evident in panel (e), that the majority of positive staining within the testis was located within the seminiferous cords. Both immature Sertoli cells and gonocytes were immunopositive as determined by their distinct morphological features. The developing epididymis (panel (f)) showed a diffuse cytoplasmic cell staining which may be nonspecific as distinctive nuclear staining was only evident in a few cells. The surrounding stromal tissue had more distinct nuclear staining.

ERβ immunolocalisation from an infant marmoset (8 weeks) is depicted in Figure 3.1 (panels g-i). At 8 weeks, the seminiferous cords of the testis clearly showed nuclear Sertoli cell and spermatogonia cell staining. There was also peritubular myoid cell staining and staining of interstitial cells (presumptive Leydig cells?). There was clear nuclear staining within the efferent duct epithelium and surrounding stroma. This staining pattern was also evident in the caput epididymis. ERβ immunostaining was evident in principal, basal and apical cells of the marmoset epididymis. Around puberty (62 weeks), it was clear that not all epithelial cells within the efferent ducts expressed ERβ (Figure 3.1 panel (j)). However, both ciliated and non-ciliated cells were immunoreactive for ERβ so that a lack of ERβ staining was not due to expression in one particular cell type. The staining within the epithelium of the caput epididymis was identical to that at 8 weeks but the underlying stromal cells appeared to be less immunoreactive for ERβ than at earlier ages (k). Panel (l) illustrates ERβ immunoexpression within the marmoset vas deferens. The thick muscular layer showed a high density of ERβ positive cells whereas the epithelium had a very weak reaction to this antibody. The testis of the adult marmoset (Figure 3.1 panel (m)) showed immunoexpression of ERβ within Sertoli cells, spermatogonia and some (but not all) spermatocytes, round spermatids but not in elongating spermatids. Interstitial cell staining of presumptive Leydig cells was also evident. Within the efferent ducts (panel n) the pattern of immunostaining was as described in panel (j) and it was more evident that both ciliated and nonciliated cells expressed ERβ. The cauda epididymis of the adult marmoset depicted a tissue in which all epithelial cell types were immunopositive for ERβ as were the surrounding stromal cells.

3.3.2 Comparison of Sex Steroid Receptor Expression in the Adult Marmoset Testis and Epididymis

Figure 3.2 is a comparison of the immunoexpression of ERβ (panels a-c), ERα (panels d-f) and AR panels (g-i) within the testis (panel a, d, g), efferent ducts (panel b, e, h) and caput epididymis (panel c, f, i) of the adult marmoset monkey. The three sex steroid receptors exhibited a different pattern of expression within the testis. ERβ had the widest cellular distribution with localisation evident within the seminiferous tubules (Sertoli cells,

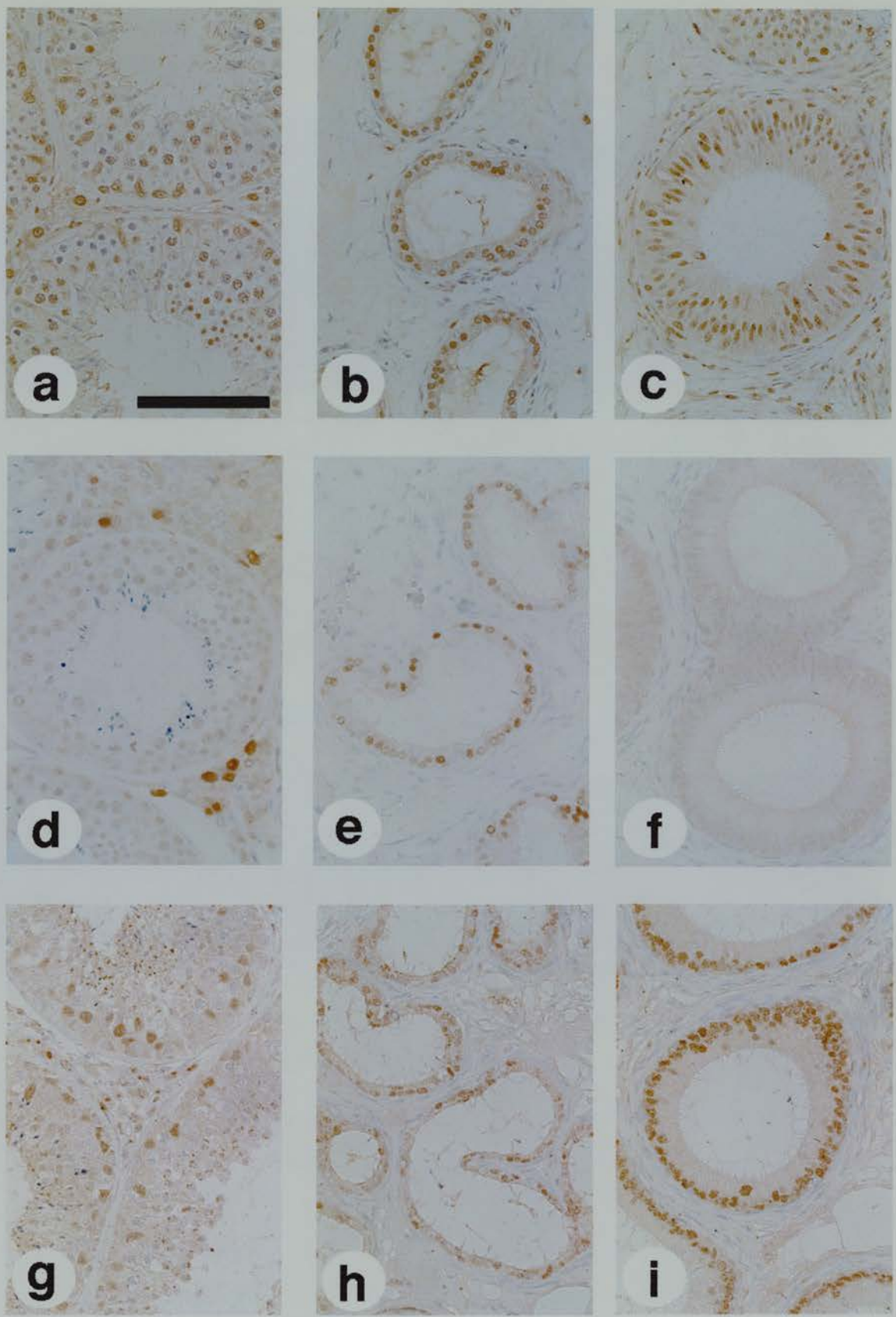

Figure 3.2 A comparison of Androgen and Oestrogen (α and β) Receptor Immunoexpression in the Male Reproductive Tract
Illustrates the comparative sites of expression of ERα (a-c), ERβ (d-f) and AR (g-i) within the testis (a, d, g), efferent ducts (b, e, h) and caput epididymis (c, f, i) of the marmoset. All images are x400 and the scale bar denotes 100μm.

spermatogonia, and some spermatocytes and round spermatids) and interstitium. ERα did not localise within the seminiferous tubules but only in Leydig cells of the interstitium (panel d). AR expression within the testis was evident in the Sertoli cells within the seminiferous tubules and within the interstitium. AR staining was also evident within the residual bodies of spermatozoa and this is believed to be a non-specific precipitate.

The efferent ducts of the marmoset were immunopositive for all three steroid receptors, and each showed a similar checker-board pattern of expression. Not all epithelial cells were positive for these receptors but both cell types (ciliated and non-ciliated) displayed some positive cells. Stromal cells surrounding the ducts were only positive for ERβ immunolocalisation (panel b). The caput epididymis showed a similar pattern of immunostaining for ERβ and AR but ERα did not localise to the epididymis of the marmoset. AR had the strongest immunolocalisation as judged by staining intensity (panel i), though ERβ immunolocalised to a higher percentage of apical cells than did AR.

In summary, ERβ expression was widespread throughout the testis and epididymis, in contrast to ERα which was expressed only in Leydig cells of the testicular interstitium and in the efferent duct epithelium. AR expression was present in the testis and efferent ducts but was expressed with highest intensity in the caput epididymis.

3.4 Discussion

The immunolocalisation of ERβ within the marmoset testis and epididymis throughout neonatal to adult life suggests that oestrogen action via this steroid receptor could have important developmental and maturational functions within these tissues. The staining pattern once clearly established (at ~ 8 weeks) did not alter significantly. Except after puberty and the initiation of spermatogenesis, it was clear later spermatocytes and early spermatids also expressed ERβ. The pattern of staining within the efferent ducts and epididymis did not alter from infancy to adulthood, similar to previous findings for ERα within the efferent ducts (Fisher *et al.*, 1997).

Finding that the steroid receptors (AR, ERα and ERβ) have some overlapping and some discrete sites of expression suggest room for interplay between the actions of androgens and oestrogens. Until recently, the endocrine control of the testis and epididymis was regarded as being androgen driven. The sites of AR expression (primarily Leydig, peritubular myoid and Sertoli cells) suggested that spermatogenesis was driven by androgens not acting directly on germ cells but indirectly via Sertoli cells (Bremner *et al.*, 1994), (Sharpe, 1994). The immunolocalisation of ERα (Fisher *et al.*, 1997) only to Leydig cells within the testis upheld

this hypothesis. However, the description of ERβ within multiple cell types of the marmoset seminiferous tubules, together with similar published data in the rat (Saunders *et al.*, 1998), (van Pelt *et al.*, 1999) suggest that the androgen dominated model of spermatogenesis may require reassessment. Within the seminiferous epithelium of the rat, ERβ has been shown to be expressed in Sertoli cells (which unlike AR expression is not stage dependent (Bremner *et al.*, 1994)) (Saunders *et al.*, 1998), spermatogonia, and later spermatocytes and early spermatids (Saunders *et al.*, 1998), (van Pelt *et al.*, 1999). These data are identical to the results obtained for the marmoset testis. The presence of a steroid receptor within the developing germ cells is unique for ERβ and is not observed for either AR or ERα. Other recent studies have localised aromatase to the testis and particularly to spermatocytes, spermatids and spermatozoa (Nitta *et al.*, 1993), (Janulis *et al.*, 1998; Janulis *et al.*, 1996a; Janulis *et al.*, 1996b) suggesting a local source of testosterone conversion to oestrogen which may allow local production and action of steroids on germ cells.

In the rat, ERβ has been identified in fetal/neonatal gonocytes (Saunders *et al.*, 1998) and the data shown here for 1 day old marmoset would also support these findings. Immature rat Sertoli cells are also known to synthesise aromatase (Weniger, 1993), again suggesting a role for oestrogen in the early development of the testis and germ cells. A recent study of ERβ expression within the rat located ERβ mRNA to gonocytes of fetal day 16 and postnatal day 4 testes (van Pelt *et al.*, 1999). Prior to puberty and the onset of spermatogenesis at days 11-15 postnatal, the only germ cells that produced a strong ERβ signal were spermatogonia. In the adult testis, ERβ mRNA was localised to pachetyne spermatocytes at stages VII-XIV and in round spermatids from stages I-VIII. This study did not find any signal within the interstitium and only weak signals in adult Sertoli cells and spermatogonia (van Pelt *et al.*, 1999). This study supports a role for oestrogen in germ cell development and maturation.

Within the epididymis, the efferent duct epithelium is the only region that localises all three steroid receptors. The luminal fluid in the efferent ducts is high in testosterone that can be converted to dihydrotestosterone or oestrogens. The principal cells of the epididymis contain 5α-reductase and spermatozoa travelling through these ducts and the epididymis have been shown to express aromatase (Janulis *et al.*, 1996b). This suggests that there is a strong interplay between androgens and oestrogens within the male excurrent duct system. Both AR and ERβ are expressed throughout the epididymis but the remainder of the epididymis does not express ERα which is consistent with previously published data (Fisher *et al.*, 1997). AR has a much more intense signal than ERβ within the caput epididymis. The pattern of staining for these two receptors within the epididymis is very similar and it would be of great interest to determine whether or not these receptors can dimerize under physiological conditions. An

earlier study examined the expression of ERα and ERβ in the excurrent ducts of the adult rat using immunocytochemistry (ERα only) and RT-PCR (Hess *et al.*, 1997) and demonstrated that the level of ERα mRNA was found to be 3.5 times higher per milligram of total RNA than rat uterus. This study also located ERβ to all the different regions of the epididymis including the efferent ducts and the vas deferens and prostate (Hess *et al.*, 1997). The results for the marmoset show that ERβ is expressed in a far wider range of cell types than is either ERα or AR. The only sites where all three receptors were expressed were the Leydig cells of the testis and the efferent duct epithelium. It is known that the two ERs form heterodimers with each other but it is not yet known whether or not the ER can interact to form heterodimers with AR. This could influence steroid interactions in Leydig and Sertoli cells within the testis and the efferent ducts and epididymis.

In summary, the discovery of ERβ has broadened the number of oestrogen target cells within the male reproductive tract. Particularly in the testis, the discovery of a steroid receptor expressed in germ cells may have far reaching implications for our current understanding in the control of spermatogenesis. Similarly, the wide expression of ERβ (but not ERα) within the epididymis indicates that this organ may not be solely an androgen dependent target and may suggest an interaction between the effects of androgens and oestrogens. The efferent ducts are known to be an important site of ERα expression but it should not be forgotten that both ERβ and AR are also expressed in this tissue and must have roles to play in its function.

Chapter 4 Aquaporin-1 Immunoexpression in the Efferent Ducts of the Rat and Marmoset

4.1 Introduction

As shown in Chapter 3 and previous studies (Fisher *et al.*, 1997) the efferent ducts are a site of oestrogen receptor expression. Oestrogen receptor-α expression is particularly high in the efferent ducts and is on a par with levels observed in the uterus. This highlights the likely relevance of the efferent ducts in oestrogen action and why studies of the role of oestrogen in male reproduction and the consequences of abnormal oestrogen exposure are important. At the outset, these studies aimed to identify functional marker/s of efferent ducts which were oestrogen regulated. Such a marker would allow a comparison between compounds regarded as potent (e.g. high doses of DES or ethinyl oestradiol) or weak (e.g. environmental) oestrogens. As the efferent ducts of the male reproductive tract are embryologically related to the kidney, and in particular the proximal convoluted tubule, a likely candidate was a recently identified water channel protein called Aquaporin-1 (AQP-1). This chapter describes a preliminary study to determine whether AQP-1 was expressed in the efferent ducts during the postnatal development of the efferent ducts in the rat and marmoset. Determining whether AQP-1 was expressed at this time point was important as many of the later studies focus on this time period.

The efferent ducts of the male reproductive system and the proximal tubules of the kidney are two sites which are responsible for a high rate of fluid reabsorption. The movement of water across most biological membranes is attributed to simple diffusion through the lipid bilayer. However, there are a number of specialised cell membranes which show a much higher water permeability than is accountable for by diffusion alone. The epithelia lining the efferent ducts and the proximal tubules exhibit a high rate of solute flux-dependent reabsorption of luminal fluid. Initially the high absorption rate was explained by the transport of water across the membrane secondary to ion transport but recently a family of proteins have been discovered, termed 'Aquaporins', which are involved in fluid movement. Water channels have been localised to many cell types, including, but not restricted to, kidney proximal tubule cells, erythrocytes, selected cells in vasopressin sensitive tissues e.g. kidney collecting duct and amphibian urinary bladder (Brown, 1989), (Sabolic *et al.*, 1992).

In 1988, a water permeable channel was identified in erythrocytes and proximal kidney tubules which was related to the lens protein MIP (Major Intrinsic Protein of the lens) (Denker *et al.*, 1988; Smith and Agre, 1991). This protein was originally called CHIP-28 (Channel-forming Integral Protein of 28 KDa) but was renamed Aquaporin-1 (AQP-1) after it was shown to function as a water-selective channel by expression in *Xenopus* oocytes and reconstruction of highly purified CHIP-28 protein into proteoliposomes (Preston *et al.*, 1992) (Zeidel *et al.*, 1992). Erythrocyte AQP-1 was purified and used to raise antisera to map other AQP-1 sites by immunolocalisation (Sabolic *et al.*, 1992).

Western blotting has identified AQP-1 as one distinct band of 28kDa and more diffuse band/s between 35-60kDa which are assumed to represent glycosylated moieties (Danker *et al.*, 1988),(Sabolic *et al.*, 1992). In the kidney, AQP-1 localises predominantly to the proximal tubules and the thin descending limb of the loop of Henle in both rats and humans (Sabolic *et al.*, 1992). It is present on both apical and basolateral membranes of the proximal tubule and also on apical vesicles (Sabolic *et al.*, 1992); AQP-1 comprises 3.8% of isolated proximal tubule brush border protein (Nielsen *et al.*, 1993).

AQP-1 is a hydrophobic integral membrane protein that functions as a water channel (Skach *et al.*, 1994), although the pore through AQP-1 has been shown to be permeable to other substrates. Both ethylene glycol and glycerol, but not urea, are able to pass through AQP-1 proteins (Abrami *et al.*, 1995). The molecular pathway that solutes take through the channel has not been fully elucidated but the molecular structure of the channel has been partially determined (Jung *et al.*, 1994). Some authors predict that the structure of AQP-1 may be analogous to many ion channels. Hydropathy analysis for both MIP and AQP-1 suggested the protein consisted of six bilayer-spanning α-helices connected by 5 loops (A-E) (Preston *et al.*, 1992). Both the amino- and carboxyl-termini were immunologically determined to be intracellular (Smith and Agre, 1991), (Nielsen *et al.*, 1993) and the model determined that loops A, C and E were extracellular and loops B and D cytoplasmic. A series of site directed AQP-1 mutants were synthesised to determine which loops were required for the functional protein (Jung *et al.*, 1994). The mutants were expressed in *Xenopus* oocytes and it was determined that cytoplasmic loop B and loop E were structural components of a single, narrow aqueous pathway through each sub-unit of AQP-1 (Jung *et al.*, 1994). Each of these loops contains a conserved NPA motif (asparagine-proline-alanine) which has been located in homologues from plant and animal species (Jung *et al.*, 1994). The tertiary structure may allow these loops to be in contact in the folded protein, forming structures which may dip into and emerge from the cytoplasmic face and extracellular face of the lipid bilayer. The meeting of these two loops at a central neck would form an hourglass structure (see Figure 4.1)(Jung *et al.*, 1994).

AQP-1 is believed to function as a multi-subunit oligomer which freeze-fracture studies have shown to be a tetramer (Verbavatz *et al.*, 1993). Further analysis by two-dimensional crystallization provided a high resolution view of AQP-1 and revealed it to be an asymmetric tetramer with central depressions extending deep into both the cytoplasmic and extracellular surfaces (Walz et al, 1994), thus supporting the hour glass model.

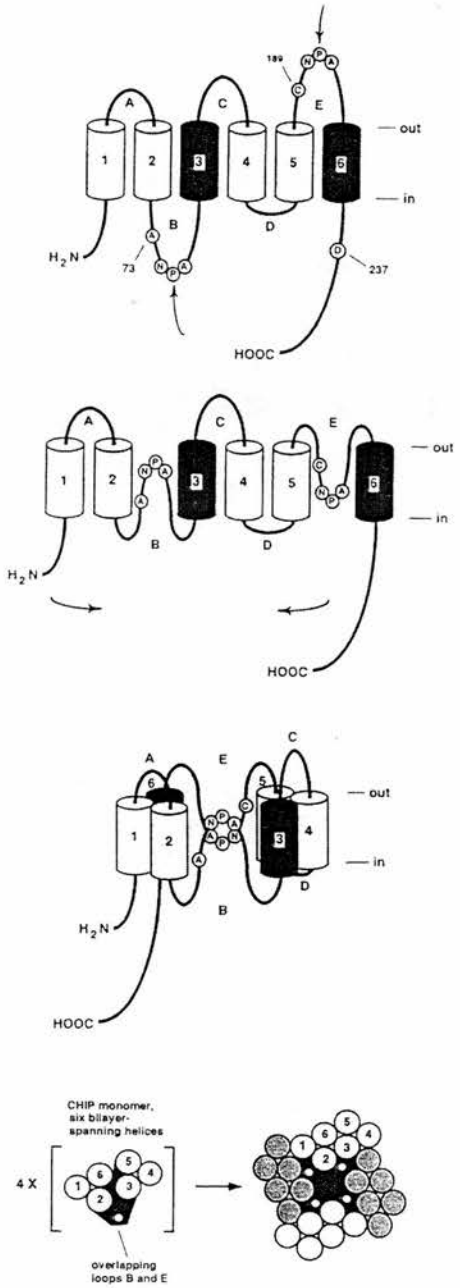

Figure 4.1 Diagram of Aquaporin-1 Protein Structure and Folding

Representing a model of how the AQP-1 protein folds to form a water transporting pore termed, the hourglass model. The final image represents the tetrameric protein structure which is believed to comprise the functional protein (Jung *et al.,* 1994).

A recent study examined the sites of AQP-1 expression developmentally by both immunocytochemistry and *in situ* hybridisation from fetal day 14 in the rat to maturity (Bondy *et al.*, 1993). This paper described three patterns of AQP-1 expression. Strong expression was found in the haematopoetic tissues and kidneys of mature rats with strong staining only observed after birth. AQP-1 mRNA was found to be abundant in the choroid plexus epithelium throughout fetal life to maturity. Messenger RNA was also transiently expressed in the periosteum, heart, vascular endothelium and cornea during

fetal life (Bondy *et al.*, 1993). The ontogeny of kidney and erythrocyte AQP-1 coincides with the ability of the kidneys to concentrate urine, suggesting that AQP-1 promotes water resorption in the proximal nephron and provides red cell osmoregulation required for passage through the hypertonic medulla of the kidney. The presence of AQP-1 in the choroid plexus suggests that AQP-1 mediated water transport contributes to the secretion of cerebrospinal fluid (Bondy *et al.*, 1993). This study demonstrates the wide variety of tissues which AQP-1 is important, not only in adults but also developmentally. This suggests there must exist complex mechanisms to regulate the expression of AQP-1. The study by Bondy *et al.*, (1993) did not investigate the reproductive system to determine whether AQP-1 was expressed in fetal / neonatal / adult life.

As described in the literature review of this thesis, some regions of the male reproductive tract are embryonically related to the kidney and the efferent ducts exhibit a similar resorptive capacity to the kidney nephron. The efferent ducts are responsible for reabsorbing a high percentage of the fluid which enters from the rete testis. Additional fluid resorption occurs along the epididymis adding to sperm concentration. The non-ciliated cells of the efferent ducts are morphologically similar to the cells of the renal proximal tubule to which they are embryologically related (Brown *et al.*, 1993). AQP-1 was immunolocalised in the efferent ducts of the adult male rat. AQP-1 was localised to the apical and basolateral membranes of the non-ciliated cells but no AQP-1 immunostaining was observed in the ciliated cells.

The studies described in this chapter re-examined the sites of AQP-1 immunolocalisation, not only in the adult rat but also at specific time points from late fetal / early neonatal, pubertal to early adulthood in both the rat and marmoset, enabling determination of whether AQP-1 is a potential functional marker of the excurrent ducts during earlier developmental stages. The aim was to establish whether or not AQP-1 expression was evident before or around the time of puberty when fluid flow from the testis increases, and whether this channel was evident in primate efferent ducts or any other regions of the male excurrent duct system.

4.2 Experimental Procedures

4.2.1 Tissue collection

Tissues from untreated control rats were obtained on fetal days 17.5 - 20.5 and on postnatal days 4, 8, 10, 15, 18, 25, 35, 48 and 90. Animals were killed as described in Section 2.1 and tissues were fixed in Bouin's fluid as described in Section 2.3.3. These tissues were processed for paraffin embedding as previously described (Section 2.3.3). Marmoset tissues were collected with the testis and epididymis attached. The tissue was classed as being from either neonates, infants, prepubertal or adults (aged 1 day, 8-24, 54-

62, 92-112 weeks, respectively; n= 2-4 per group). These tissues were collected from control animals from a different experiment and had been processed into paraffin blocks.

4.2.2 Antibody Production

The AQP-1 antibody was a gift from Professor Dennis Brown (Harvard Medical School, Boston). The immune serum was prepared from human erythrocytes. AQP-1 was isolated from potassium-iodide stripped erythrocyte ghosts (Van Hoek and Verkman, 1992). The antibody was then produced as described by Sabolic *et al.*, (1992). Briefly, 0.25 mg of purified AQP-1 was injected subcutaneously with Freunds complete adjuvant into New Zealand white rabbits. Boosters of 0.1mg protein were given every three weeks with Freunds incomplete adjuvant. Sera from bleeds were checked for specific antibody production by immunocytochemistry and Western analysis (Sabolic *et al.*, 1992). In the present studies AQP-1 antiserum and its pre-immune control were used at a dilution of 1:500.

4.2.3 Western Blot Analysis

Western blots were performed using 75µg of adult efferent duct and kidney proteins that were separated using 12% acrylamide SDS-PAGE blotted onto PVDF membrane and probed with AQP-1 antiserum and pre-immune serum at 1:5000. The methods are fully described in Chapter 2 (sections 2.6-2.8) using ECL detection.

4.2.4 Immunoctyochemistry on paraffin tissue sections

Immunocytochemistry was performed as described in Section 2.4.1.

4.2.3 Photomicroscopy

The slides were photographed as described in Section 2.5.1.

4.3 Results

4.3.1 Western Blot Analysis of AQP-1

The result shown in Figure 4.2 demonstrates the specificity of AQP-1 antibody in recognising the 28kDa protein in both adult rat kidney and efferent duct protein samples. The higher band represents the glycosylated form of the AQP-1 protein.

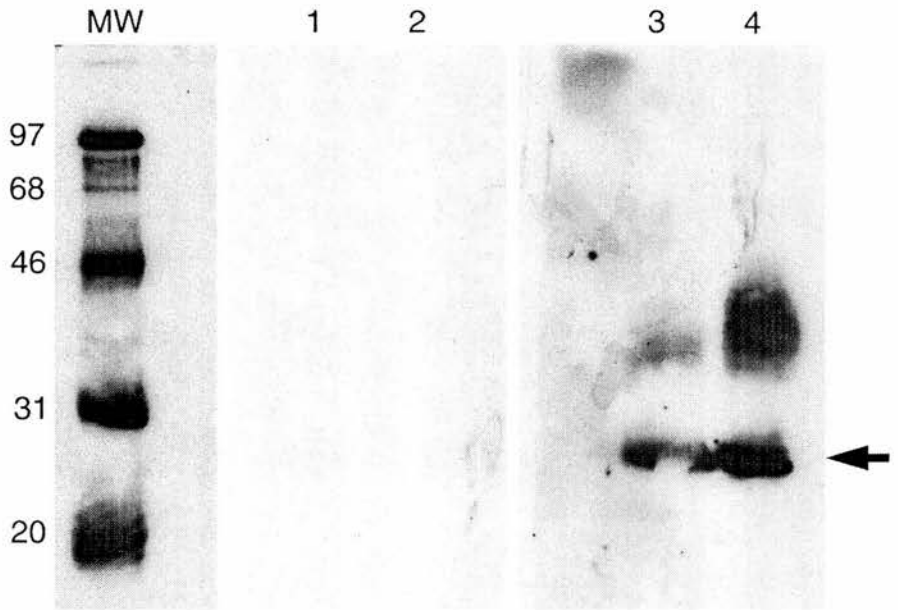

Figure 4.2 Aquaporin-1 Detection by Western Blot

Western Blot analysis of AQP-1 in adult kidney (lane 1,3) and efferent duct (2, 4) protein. Lanes 1 and 2 represent blots incubated with pre-immune serum while lanes 3 and 4 were incubated with AQP-1 antisera. The arrow indicates 28 KDa.

4.3.2 Developmental Pattern Of AQP-1 Immunoexpression in the Rat

The results shown in Figure 4.3 are digital photomicrographs. A positive control tissue section of rat kidney is shown in panel (a) and depicts AQP-1 immunostaining (brown precipitate) within the thin descending limb of the loop of Henle, the kidney collecting ducts also expressed AQP-1. It is evident from the figure that AQP-1 was expressed in the efferent ducts at all ages from fetal life onwards. During fetal life AQP-1 immunoexpression was evident from day 17.5 (earlier time points were not assessed) and persisted throughout the rest of fetal life (days 17.5 and 19.5 are shown in panels c and d respectively; days 18.5 and 20.5 are not shown). Note also that the lumens of the developing efferent ducts were patent even at this early time point. In early postnatal life (day 4, Figure 4.3 (e)), it was not yet possible to distinguish different cell types within the epithelium and all cells had a columnar appearance.

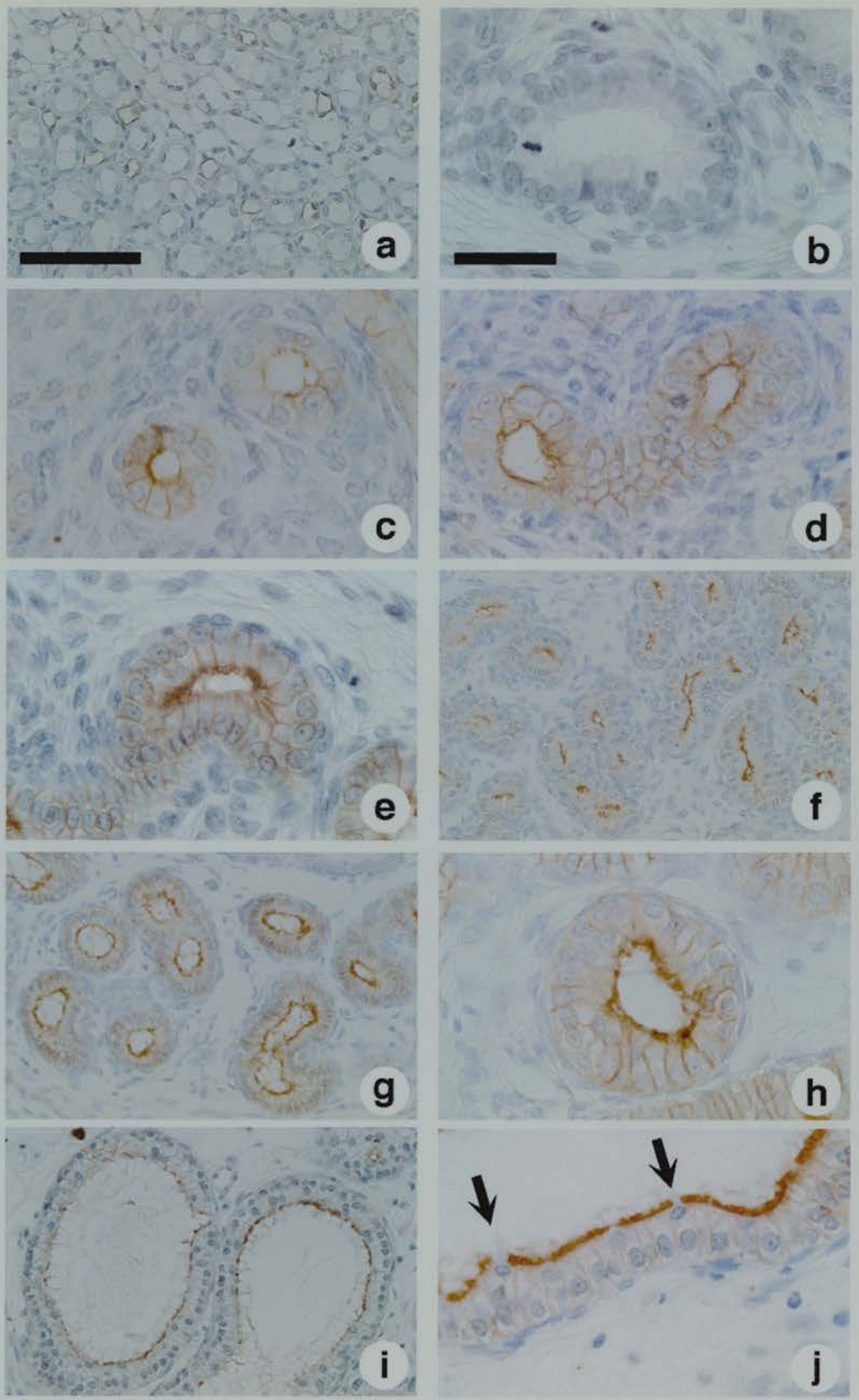

Figure 4.3 Aquaporin-1 Immunolocalisation in the Rat
Immunoexpression of AQP-1 in the efferent ducts of the rat from fetal life to adulthood. A positive control tissue section of rat kidney (a) and a negative control (b) of postnatal day 4 efferent ducts are shown. Representative patterns of immunostaining are shown for rats aged (c) fetal day 17.5, (d) fetal day 19.5, postnatal days (e) 4, (f) 10, (g) 18, (h) 25 and (i, j) 90 days.panels b, c, d, e, h, j are shown at a higher magnification of x1000 using oil immersion. All other panels are shown at x400. The scale bars in (a) and (b) denote 100 and 20μm respectively. The arrows (j) indicate ciliated cells.

AQP-1 immunolocalisation appeared as a thick apical band staining the brush border of the epithelial cells though there was also immunostaining of the basolateral membrane. A similar section incubated with pre-immune serum as a negative control, revealed no immunostaining (panel b). The situation was almost identical at days 10, 18 and 25 except that it was possible to see that the epithelial cells were differentiating such that ciliated cells could be identified by the apical position of their nuclei (Figure 4.3 (f, g, h)). At day 35 (late puberty) the ciliated cells were absent from AQP-1 immunostaining (not shown). From day 18, the lumen of the efferent ducts increased in diameter until adulthood consistent with the age at which production and flow of STF has been shown to commence (Setchell *et al.*, 1994). The pattern of AQP-1 immunostaining is identical in adulthood (Figure 4.3 (i)). The morphology of the adult epithelium is clearly shown in Figure 4.3 (j), the high columnar epithelium displayed an extensive brush border. The apical nuclei of the ciliated cells were clearly visible as were the basal nuclei of the non-ciliated cells. It was quite clear that ciliated cells did not immunoexpress AQP-1 whereas non-ciliated cells showed high levels of expression (panel j; arrows). The testicular parenchyma and the epididymis were never immunopositive for AQP-1 (not shown).

4.3.2 Developmental Pattern of AQP-1 Immunoexpression in the Marmoset

The results of AQP-1 immunolocalisation in the marmoset monkey were broadly similar to the rat (Figure 4.4). A section of adult marmoset kidney was immunostained with AQP-1 as a positive control (panel a). The staining was identical to rat kidney with AQP-1 localising to the collecting ducts and the thin descending loop of Henle; panel (a) shows the collecting ducts. On day one postnatal the developing efferent ducts showed a similar pattern of expression to the fetal rat (Figure 4.4 (b)). The fixation of this tissue is not as good as the rat and many of the ducts have collapsed but there were a few which clearly had a patent lumen. By eight weeks the efferent duct lumens were more obvious and the brush border was clearly immunostained with AQP-1 as were the basolateral membranes (Figure 4.4 (c)). The situation was identical at 17.6 weeks of age (panel d). The epithelium of the marmoset efferent ducts showed a more intense reaction to the AQP-1 antisera than did the rat tissue. The brush border displayed intense immunoreaction and the cytoplasm of many of the epithelial cells were also heavily immunostained. At 62 weeks (peripubertal; Figure 4.4 panel e) the efferent duct lumen had expanded and the intracellular immunostaining appeared to have increased. It was not possible to locate an apical cell membrane (i.e. from a ciliated cell) which was not immunoreactive for AQP-1. The situation was identical in the adult marmoset (92 weeks, panel f).

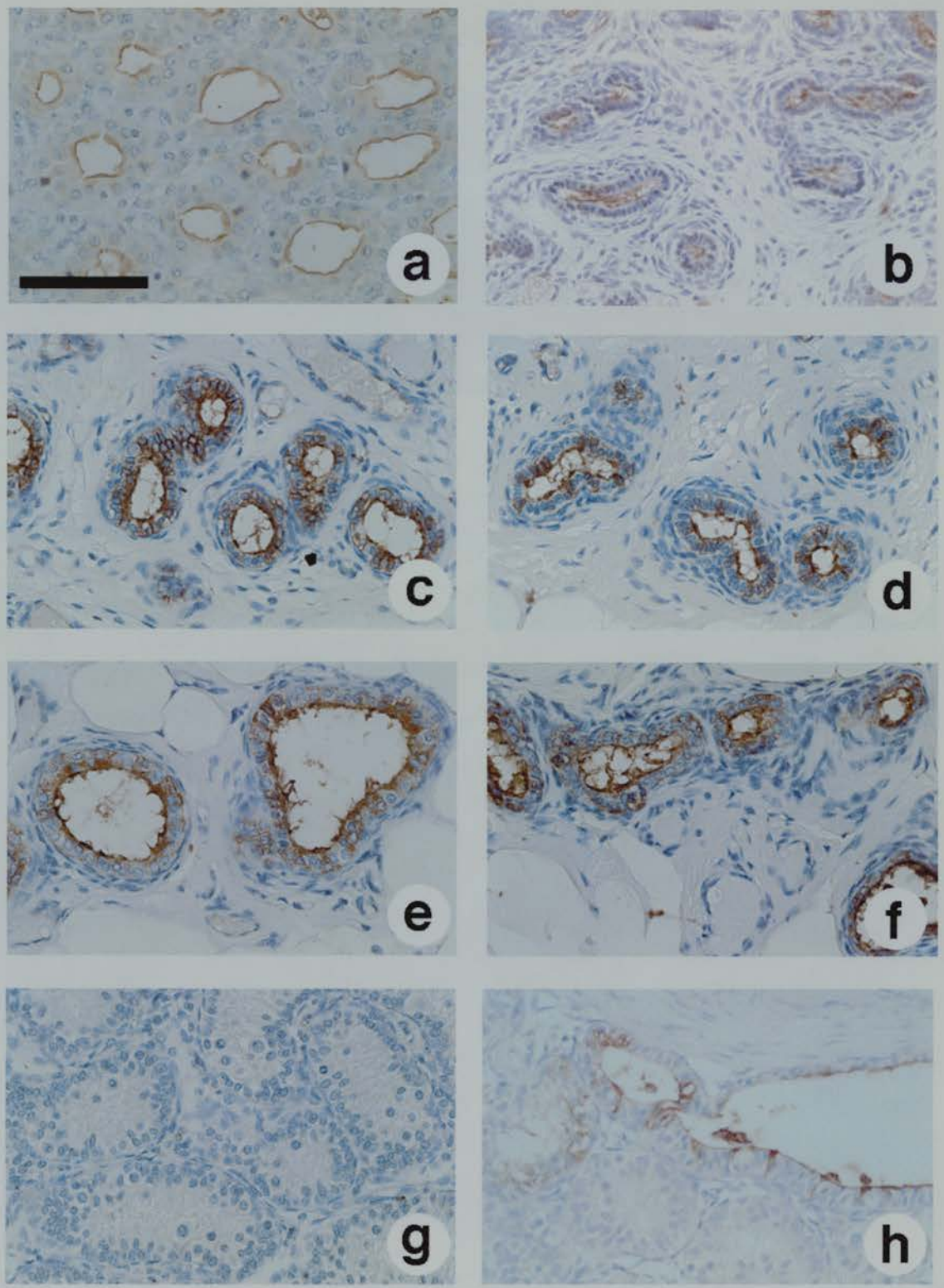

Figure 4.4 Aquaporin-1 immunolocalisation in the Marmoset
Immunolocaisation of the efferent ducts from marmoset monkey from the perinatal period through to adulthood. A positive control section of marmoset kidney is shown in panel (a). AQP-1 staining is shown for animals ages (b) one day, (c) 8 weeks, (d) 17.6 weeks, (e) 62 weeks and (f) 92 weeks. Panel (g) depects the testis and (h) rete testis at 8 weeks. All photomicrographs are shown at x400 and the scale bar denotes 100μm.

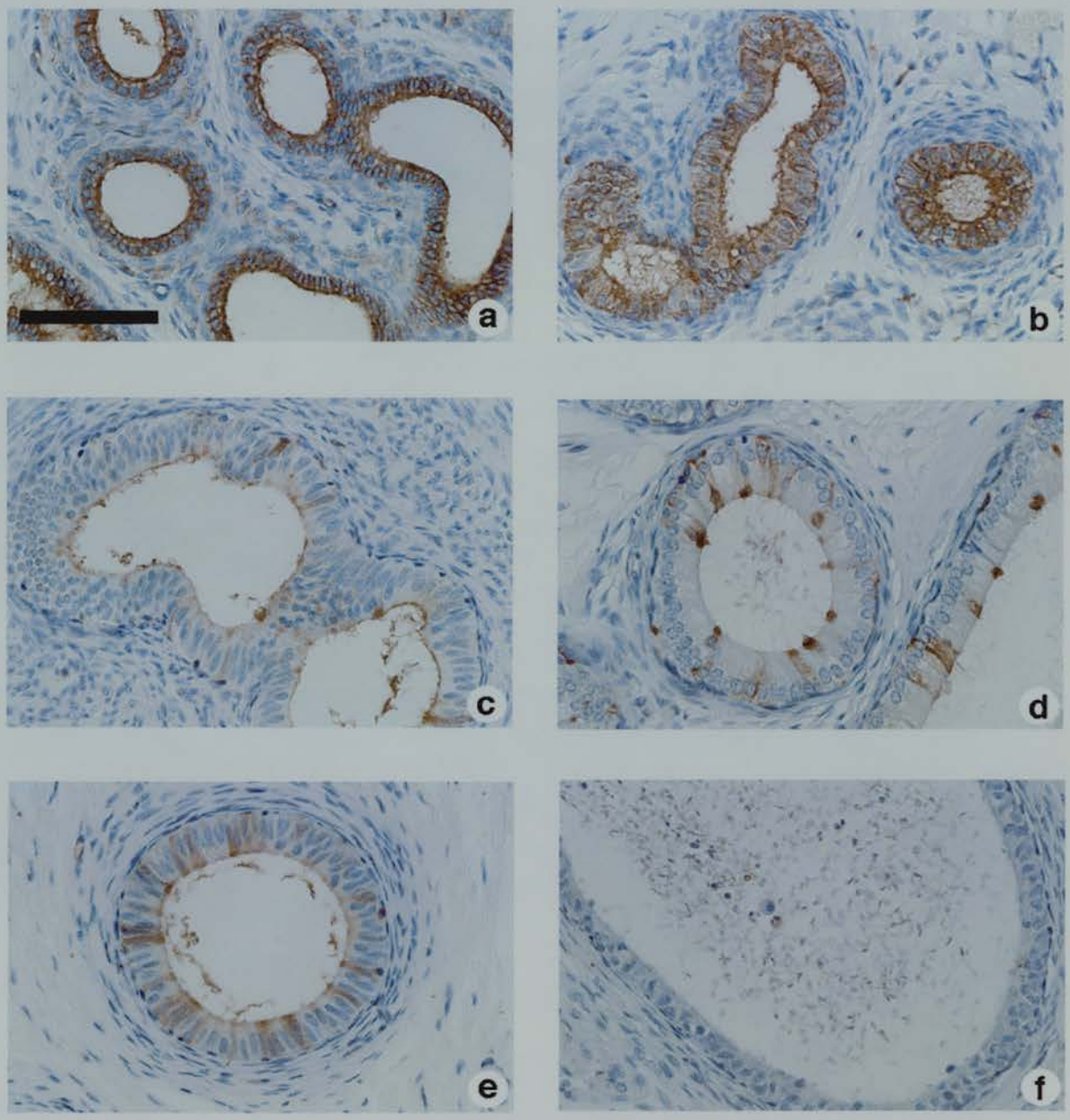

Figure 4.5 Aquaporin-1 Immunocytochemistry in the Marmoset Epididymis
Immunocytochemical staining of the marmoset epididymis at 8 weeks (a; initial segment), 17.6 weeks (b; initial segment), c (caput region), 62 weeks (d; caput, f; cauda). All images are shown at x400 and the scale bar denotes 100μm.

As in the rat, there was no expression of AQP-1 within the seminiferous tubules or interstitium (Figure 4.4 (g)). However, where cross-sections of the rete testis were evident (neonatal and peripubertal animals) there was a narrow apical band localised to the rete testis and this extended to the basolateral membrane in some cells (panel h).

Unlike the rat, however, the marmoset epididymis was immunopositive for AQP-1 when assessed at 8, 17.6 and 58.7 weeks of age (Figure 4.5). Aside from the efferent ducts, the most intense immunoreaction was within the initial segment of the epididymis (Figure 4.5 (a,b); ages 8 and 17.6 weeks respectively). In the caput epididymis of the younger animals (17.6 weeks) there was a fine apical band of immunostaining along the epididymal epithelial cells (panel (c)) but at puberty (58.7 weeks; panel (d)) a specific subset of principal cells termed apical cells showed a strong immunoreaction along their apical and basolateral surfaces. This was still evident but less common in the corpus epididymis (not shown) with some staining evident in the cauda neonatally (Figure 4.5 (e)). Around puberty, there was no epithelial staining of the cauda epididymis (panel f).

4.4 Discussion

The possibility that the water channel AQP-1 may be a potential functional marker of the efferent ducts was investigated in these experiments. The efferent ducts are an important site of ER action and their major function is fluid resorption. The strong expression of AQP-1 in the efferent ducts from fetal life through to adulthood strongly suggests it is an important factor in fluid dynamics within this region. The Western blot demonstrates the specificity of this antibody in detecting the AQP-1 protein (Figure 4.2).

The presence of AQP-1 during fetal and early neonatal life was unexpected as fluid flow through the rete testis and efferent ducts is generally considered as being coincident with puberty and the production of seminiferous tubule fluid (Setchell *et al.*, 1994). The present results imply that fluid is flowing through these ducts earlier in development than the published data would suggest. The observation that the ductules are always patent, even in fetal life, supports this interpretation. It was evident however that the lumens of the efferent ducts expanded in rats from day 18 postnatal onwards and continued to increase in diameter until adult life presumably indicative of increased fluid flow from the testis at puberty.

AQP-1 localisation in the rat was limited to the efferent ducts with no immunolocalisation to either the testis or the epididymis. During the neonatal period before the ciliated epithelial cells had differentiated in the efferent ducts, all the epithelial cells showed a strong immunoprecipitate along the apical brush border and a weak reaction in the lateral and basal membranes. Between day 18-25 postnatal, it was clear that ciliated cells did not

express AQP-1. These finding are in agreement with those already published for the adult rat which indicate that only non-ciliated cells express AQP-1 (Brown *et al.*, 1993).

The immunolocalisation of AQP-1 within the efferent ducts of the marmoset showed broadly the same pattern of expression as the rat, except the immunoprecipitate was more intense and in some animals cytoplasmic staining was evident. It appeared that all epithelial cells were immunostained, suggesting that either all epithelial cells within the marmoset epididymis express AQP-1 or that the precipitation reaction to the antibody was so intense that it was not possible to distinguish any unstained cells. The marmoset also showed positive immunostaining in other regions of the excurrent duct system, though in the rat, the testis showed no staining for AQP-1. The rete testis was positive and there was an apical layer of staining along the rete epithelium and there were also some specific cells that had a stronger apical staining and also basolateral immunolocalisation. This might indicate that the cell types within the rete testis are not homogeneous and that different cell types exist and perform specific functions such as fluid resorption.
The transitional zone at the terminal region of the seminiferous tubules also displayed some positive cells.

The epididymis of the marmoset, but not the rat, also showed some immunolocalisation of AQP-1 throughout development. In general the most intense immunoexpression was in the efferent ducts and initial segment of the epididymis where a strong apical immunoexpression was evident along the epithelium. Within the caput epididymis apical/narrow cells were staining and the distribution of this cell type decreased towards the cauda region of the epididymis with hardly any cells (probably clear cells) staining in this region. This would suggest that AQP-1 is differently distributed in the marmoset. The increase in staining density may be a true reflection of the sites of AQP-1 expression in the marmoset or it may indicate a cross reaction with another protein by the polyclonal antiserum used in these studies

The AQP-1 staining on the apical surface of the non-ciliated cells within the efferent ducts supports the morphological findings that the resorptive apparatus is confined to this cell type (Brown *et al.*, 1993), (Hermo and Morales, 1984), (Goyal and Hrudka, 1980). These results update the model of fluid resorption within the efferent duct that was proposed by Ilio and Hess (1994). This model suggested that water resorption occurs secondary to ion transport, and during transit through the efferent ducts the fluid concentration of Na^+ is lowered whilst that of H^+ is increased indicating the presence of ion channels within the efferent duct epithelial membrane (Ilio and Hess, 1992). The immunolocalisation of AQP-1 to the apical membrane implicates this protein as a primary candidate for the absorption of water into nonciliated cells. However AQP-1 does not transport electrolytes (Preston *et al.*, 1992), therefore other channels must exist to facilitate the entry of Na^+ and maintain

the osmotic environment. How water exits the cell is not clear, but this may occur partly secondary to ion transport as a Na^+K^+-ATPase has been localised to the basal and basolateral membrane of the efferent ducts (Byers and Graham, 1990), (Ilio and Hess, 1992). There is some immunoreactive AQP-1 along the basal and basolateral membrane of the non-ciliated cell but the staining is less intense than at the apical surface suggesting that AQP-1 is of less importance in water transport across this membrane.

The exact time of onset of AQP-1 expression was not determined in this study but as the efferent ducts develop from the mesonephric tubules which do function as a kidney in fetal life one might hypothesise that it is expressed throughout its development and differentiation. Studies would have to be performed to test this possibility. It has been proposed that during sexual differentiation, testosterone from the fetal Leydig cells may be transported along the Wolffian duct by fluid flow rather than by simple diffusion (Tong *et al.*, 1996). The presence of AQP-1 in the fetal ducts at this time supports this train of thought and raises the question as to what induces AQP-1 expression in fetal life. It has been shown in the fetal lung that maternal corticosteroids induce expression of AQP-1 (King *et al.*, 1996). This finding together with strong expression of ER-α and AR in the efferent ducts, raises the possibility that sex steroids are involved in the expression of AQP-1 in the efferent ducts. This possibility will be addressed in Chapter 6.

Chapter 5 The Effect of Neonatal Exposure to Oestrogenic Chemicals on Testis Weight and the Morphology of the Rete Testis

5.1 Introduction

The experiments described in Chapters 5 and 6 are based on investigating alterations to the morphology of the rete testis and efferent ducts of the rat reproductive tract after neonatal exposure to oestrogenic compounds. These chapters are based on results from the same treatment groups and the background information regarding the rationale for these studies is detailed below and applies to both these chapters.

The studies examined the effects of oestrogen administration during the neonatal period. As discussed in the literature review, the neonatal period is important for the normal development of the reproductive system. In particular, it is the period of major Sertoli cell multiplication, which determines the adult spermatogenic output of the testis, and also a time of growth and development of the male excurrent duct. During this time accessory organs are still developing. Studies in the literature have shown that this time point is very sensitive to disruption by exogenous steroids. The aim was to examine the effects of treating rats during this time period in order to determine possible roles of oestrogen within the excurrent ducts and also to determine whether any weak 'environmental' oestrogens could induce any detectable effects similar to those induced by more potent oestrogens. These studies will aid understanding of the role of oestrogen within the developing reproductive tract and determine whether any information can be gained from animal models to elucidate the role of environmental chemicals in the observed changes to male reproductive health discussed in the literature review.

In essence, the basic strategy was to use high doses of DES (starting at 10μg/injection) administered subcutaneously to neonatal animals (on alternate days from days 2-12 postnatal) to determine the effects of high dose oestrogen exposure on the excurrent duct system. DES was used in these studies as it is known to be a potent oestrogen which can induce adverse effects on the male reproductive tract of both humans and laboratory animals (see Sections 1.2.6 and 1.7.3). In essence this compound acted as a positive control. Two lower doses (1μg and 0.1μg/injection) were used to determine whether there was a dose-response for any of the observed effects and whether it was possible to establish a no observable effect level (NOEL). A cohort of animals were treated with ethinyl oestradiol to ensure the effects induced after administration of the DES were comparable to those induced by the administration of another synthetic oestrogen (ethinyl oestradiol) at the same dose (10μg/injection).

It was also necessary to ensure that any effects observed were due to the direct actions of oestrogen acting on the target organs and were not secondary effects from oestrogen acting via the hypothalamo-pituitary axis. To determine this, a GnRH antagonist was administered to a cohort of animals to suppress LH/FSH secretion to allow us to determine if this affected the excurrent ducts. This study would differentiate between the effects of gonadotrophin action and those induced as a direct result of oestrogen exposure. The effect of tamoxifen administration was also examined as it may act as an oestrogen receptor antagonist and would also allow the effects of decreasing oestrogen levels within the developing neonatal rat to be assessed. Finally, cohorts of animals were treated neonatally with one of the following weakly oestrogenic compounds namely, octylphenol, bisphenol A, parabens or the phytoestrogen genistein, to determine whether these induced any of the effects observed after DES exposure. In order to examine the nature, severity and reversibility of the effects, groups of treated animals were examined at various time points during prepubertal, postpubertal and adult life (10, 18, 25, 35 and 75 days).

The lack of known oestrogen-regulated endpoints in male animals encouraged the use of a histology based approach to identify changes in the excurrent ducts between control animals and those which had been treated neonatally with either potent or weakly oestrogenic compounds. This chapter assesses changes in rete testis morphology induced by oestrogen treatment so it is necessary to review the literature describing normal rete testis morphology and its development.

The rete testis is not a well-studied structure. Unlike the testis, which is responsible for producing sperm, or the epididymis, which is important for sperm storage and maturation, the rete testis does not appear to have such a vital biological function (as judged by the paucity of published reports!). However, were it not for the rete testis the sperm and testicular fluid would never reach the epididymis, so although many authors consider this structure as a conduit for sperm transport, this would appear a rather vital function, enabling sperm to reach the epididymis and allowing sperm maturation to occur. The function of the rete testis or the need for this structure is not clear but this structure has an intratesticular region that then passes through the tunica albuginea into an extra-testicular region and, developmentally, it is the region of urogenital union. After the onset of spermatogenesis this structure appears to function a bit like a roundabout. The sperm leaving the testis enter the rete testis from different seminiferous tubule junctions where they are all mixed and then exit out of the testis and enter the excurrent duct system.

Basic anatomical information regarding the rete testis has been described in the literature review (Section 1.3.3). The rete testis of the rat has both intra- and extra- testicular regions,

which has raised an interesting embryological question as to the origin of these structures. The intratesticular rete forms from the gonadal blastema which consists of a great number of epithelial and germ cells (Roosen-Runge, 1961). It arises on day 12 of fetal life by proliferation of the coelomic epithelium which covers the ventro-medial aspect of the mesonephric ridge. Development of the rete occurs together with the sex cords as they are linked at their terminal regions. On day 19 p.c., the rete strands show the first signs of lumen formation (Roosen-Runge, 1961). By day nine postnatal the intratesticular rete has completely anastomosed within the rete structure and sex cords and has essentially merged into a single cavity. During fetal development the rete testis epithelium shows a similar composition and structure to the sex cords but by day 19 p.c., when lumen formation occurs, it has become a simple low columnar epithelium containing occasional germ cells. During postnatal development the epithelium gradually reduces in height and germ cells are no longer observed. The reduction in rete testis epithelial cell height is most evident 3-4 weeks after birth when spermatogenesis is initiated. This is also the time when the greatest increase in rete testis lumen size occurs (Roosen-Runge, 1961).

The origin of the extratesticular portion of the rete testis is less certain. Prior to the connection between the gonad and mesonephros there exists an aggregation of cells between these two structures on day 13 of fetal life (Roosen-Runge, 1961). Bridges of cells can be observed between these two structures which are morphologically different from cells of either structure. By the end of day 14 p.c. there are rudiments of an extratesticular rete (rete blastema). By 15.5 p.c. many fine basement membranes can be observed which vaguely outline a network of strands within the rete blastema. These basement membranes become progressively thicker. Canalization of the extratesicular rete does not occur until day 20 p.c. and continues after birth (Roosen-Runge, 1961).

Very few abnormalities involving the rete testis have been described, except those induced experimentally by efferent duct ligation. This is often performed in studies examining the composition of seminiferous tubule fluid and few studies exist which have examined the effects of ligation on the rete testis itself. Efferent duct ligation in the rat leads to the accumulation of fluid behind the ligature that continues in a linear manner for up to 30 hours (Setchell, 1970). The build up of fluid often induces dilatation of the rete testis which aids researchers in cannulating the rete but few have studied the eventual morphological changes in the rete testis induced by this dilatation. Rete testis distension has been observed six hours after efferent duct ligation whereas dilatatory effects were not observed within the seminiferous tubules until 12 hours (Smith, 1962). The central cavities of the rete testis have been shown to undergo a marked dilatation 24 hours after efferent duct ligation with much less distension observed in the tubuli recti which are supported by connective tissue struts.

Some tubuli recti appear to be compressed by a valve-like rete septum and can resist back-pressure up to 12 or 24 hours after ligation. After this time the valves appeared to invert, which is a sign of retrograde flow from the rete testis into the seminiferous tubules (Nykanen, 1980). Although ligation studies have been used as a means of differentiating between systemic (hormonal) regulation of the epididymis and factors deriving from testicular STF (Robaire and Hermo, 1988), the effects of fluid distension alone on reproductive tract physiology have yet to be established.

5.2 Materials and Methods

5.2.1 Animals

The animals were housed and treatments administered as described in sections 2.1 and 2.2. Prior to fixation, one testis was removed and weighed after the removal of the epididymis. To examine the effect of treatment on rete morphology, tissue that had been embedded in paraffin wax (as described in sections 2.3.3 and 2.3.4) was carefully sectioned. As the rete is a diffuse, flattened structure, care was taken to ensure tissue sections from different animals were from comparable regions of the rete. Therefore, only sections that were seen to pass through the tunica albuginea and join the extratesticular portion and efferent ducts were compared. This meant carefully examining every 5-10th paraffin section using light microscopy to locate the rete testis and efferent ducts. When the rete was located, haematoxylin and eosin staining determined the quality of the cross section.

5.2.2 Haematoxylin and Eosin (H&E) Staining Protocol

Slide mounted tissue sections were deparaffinised in Histoclear for 5 min before being rehydrated through a decreasing series of alcohols (absolute, 95% and 70%) each for approximately 20 seconds. The slides were then rinsed in water and placed into Harris's haematoxylin for 5 min and then rinsed. To remove any cytoplasmic staining the slides were immersed in 1% acid alcohol for a few seconds and then washed in water. The slides were then placed in Scott's tap water until the nuclei turned blue and the slides were again washed in water. The slides were incubated briefly in eosin (5-20 seconds) and washed in water. The slides were then dehydrated through a series of increasing alcohols and cleared in xylene before being coverslipped. The tissue sections were examined microscopically and digital images were captured and analysed as described in section 2.5.1.

5.3 Results

5.3.1 Testicular Weights

Testicular size is known to be decreased after postnatal exposure to oestrogenic compounds (Fisher *et al.*, 1998) and provides a gross indication of the number of cells within each testis. Therefore testis weights were compared between control and treated cohorts at 18, 25, 35 (not

shown) and 75 days of age (Figure 5.1 A-C). All testis weight values quoted in the text represent the mean±SD mg.

The results shown in Figure 5.1A are those for animals at18 days of age. Normal control animals had testis weights of 64.3±12.2mg. In comparison with this, GnRHa treated animals showed a highly significant reduction in testis weight (22.4±3.4mg), demonstrating that inhibition of the hypothalamo-pituitary axis inhibits normal testis growth. Higher doses of DES (10μg/injection) showed a similar reduction in testis weight (24.6±2.5)mg in comparison with control. Although the response to DES was dose dependent, at the lowest concentration used (0.1μg/injection), testicular weights were similar to control animals (61.1±11.9mg). Ethinyl oestradiol (10μg/injection) and the oestrogen receptor antagonist/ agonist tamoxifen both produced similar results to the highest dose of DES and the GnRHa (30.7±2.6mg; 23.2±2.6mg respectively). These results indicate that potent oestrogens (DES, ethinyl oestradiol) and tamoxifen are capable of inhibiting normal testis growth.

Figure 5.1A also demonstrates that weak oestrogens (octylphenol, methylparabens, bisphenol A and genistein) did not cause any reduction in testis weight at this time point. However, both octylphenol treated rats and animals whose mothers were fed on a soy-free control diet showed increased testis weights (87.0±10.2mg, 82.7±18.8mg respectively). The increase in testis weight in soy-free animals suggests that either the amount of soy protein in normal animal feed is enough to cause a significant reduction in normal control testis weight, or that soy-free feed induces an increase in normal testis size. Similarly, neonatal octylphenol treatment may increase normal testis weight perhaps due to a possible anabolic action of this compound. Neonatal treatment with the phytoestrogen genistein induced a significant reduction in testis weight (74.4±7.8mg), when compared to control animals whose mothers were fed on a soy-free diet. However, the testis weights of genistein treated animals was not different from normal control animals. This may suggest that genistein treatment reduced testis size (compared to soy-free controls) but this decrease was simply returning testis weights to values comparable to normal control animals whose diet contained phytoestrogens, including genistein.

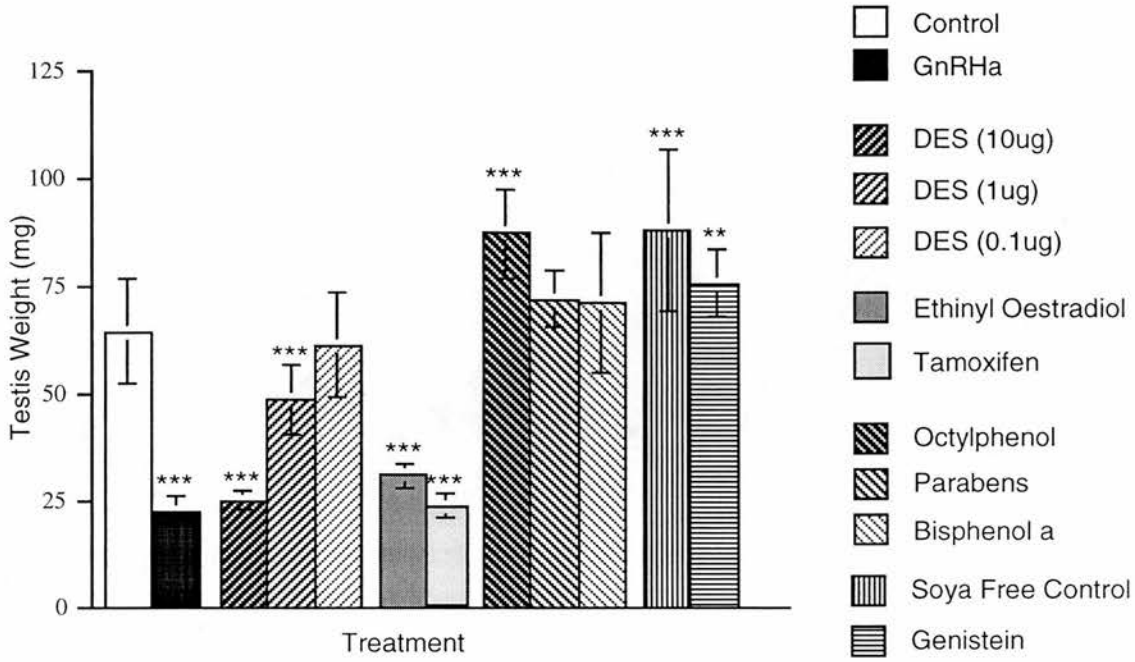

Figure 5.1A Comparison of Testis Weight (mg) at Day 18 Postnatal After Neonatal Administration of Oestrogenic Compounds

Testis weights were compared in 18 day old rats after neonatal treatment with vehicle (Control; n=11), GnRHa (n=12), DES 10μg (n=14), DES 1.0μg (n=10), DES 0.1μg (n=10), ethinyl oestradiol (n=4), tamoxifen (n=4), octylphenol (n=8), parabens (n=6), bisphenol A (n=6), soy-free control (n=13) and genistein (n=14); the doses administered were as detailed in Chapter 2 (Section 2.2). The data represents the mean ± S.D. (**p<0.01 compared with the soy-free control group; ***p<0.001 compared to the normal control group).

Figure 5.1B demonstrates testis weights for rats killed at day 25; these show a similar pattern to those described on day 18 (Figure 5.1A). The highly significant reduction in testis weight induced by neonatal treatment with GnRHa (46.6±24.6mg) was still apparent when compared with controls (165.5±27.5mg). However in comparison with day 18 data, neonatal treatment with DES (1.0μg) had not decreased in the dose-dependent manner observed at the earlier time point. The reason for this is unclear. However DES at all doses administered, decreased testis weight, although the data for DES (1.0μg) was not significantly reduced when compared to controls. Ethinyl oestradiol again significantly reduced testis weight (56.6±12.3mg), to a similar degree as GnRHa treatment. Octylphenol, at this time point did not affect testis weight in comparison with control, contrary to the data shown for day 18. The genistein (150.7±62.1mg) and bisphenol A (179.0±37.2mg) treated animals showed no change in testis weight when compared to control animals.

Testis weight at day 35 (data not shown) and in adults (Figure 5.1C) demonstrated similar changes to the earlier ages. GnRHa treatment again reduced testis weight (249.7±28mg) compared to control (759.8±90.0mg), while DES dose-dependently decreased testis weight similar to the results shown for day 18 (Figure 5.1A). Bisphenol A treatment caused no significant change in testis weight in adulthood but at 35 days of age there was a slight but significant decrease in testis weight in this group (not shown). Animals treated neonatally with genistein had marginally but significantly larger testes in adulthood (2384±100.8mg).

Figure 5.1 (A-C) illustrates that testis weights were significantly reduced at all ages after neonatal exposure to GnRHa, ethinyl oestradiol and all three doses of DES (in adulthood). As the data from the soy-free control group did not differ significantly from control animals in any parameter assessed at day 18 (except testis weight), for simplicity, at all other ages assessed, the data from soy-free control animals were pooled with "normal" control data.

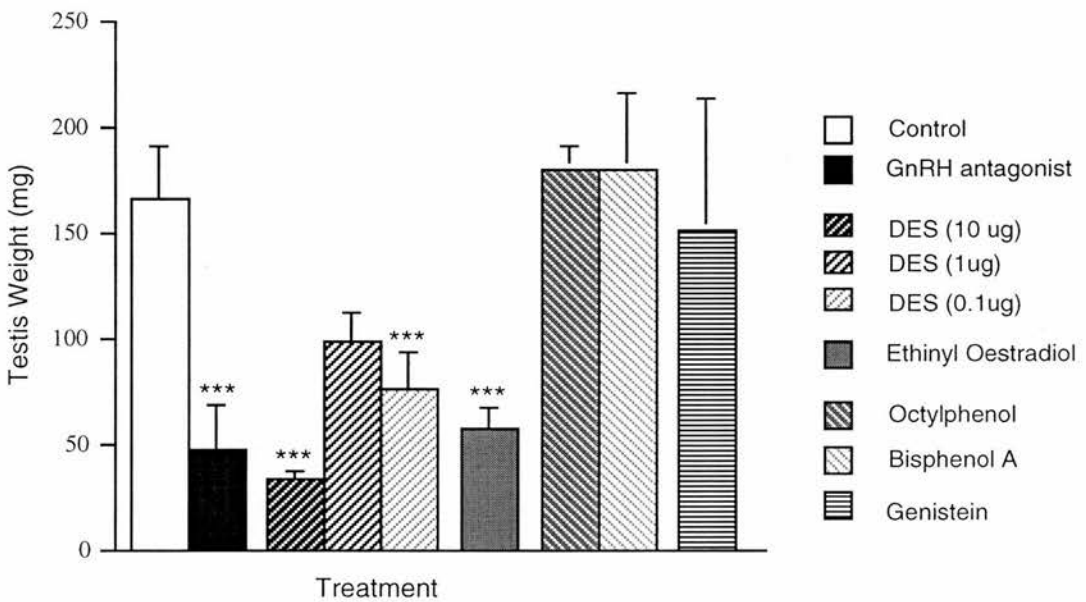

Figure 5.1B Comparison of Testis Weight at Day 25 Postnatal After Neonatal Administration of Oestrogenic Compounds

Testis weights were compared in 25 day old rats after neonatal treatment with vehicle (Control; n=12), GnRHa (n=14), DES 10μg (n=8), DES 1.0μg (n=5), DES 0.1μg (n=4), ethinyl oestradiol (n=7), octylphenol (n=6), bisphenol A (n=5) or genistein (n=7); the doses administered were as detailed in Chapter 2 (Section 2.2). The data represents the mean ±S.D. (***$p<0.001$ compared to the normal control group)

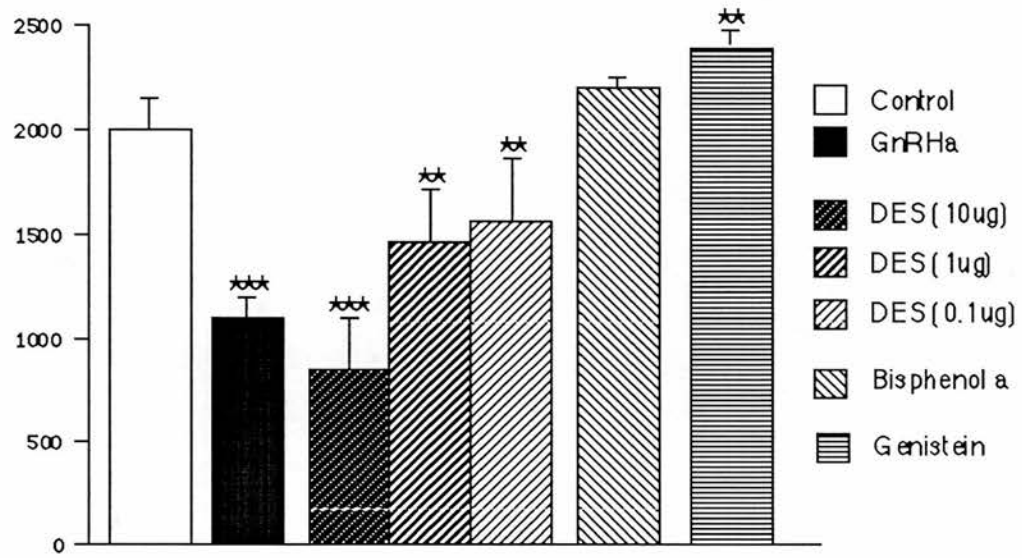

Figure 5.1C Comparison of Testis Weight at Day 75 Postnatal After Neonatal Administration of Oestrogenic Compounds

Testis weights were compared in 75 day old rats after neonatal treatment with vehicle (Control; n=6), GnRHa (n=5), DES 10μg (n=6), DES 1.0μg (n=4), DES 0.1μg (n=4), bisphenol A (n=3) or genistein (n=5); the doses administered were as detailed in Chapter 2 (Section 2.2). The data represents the mean ± S.D. (**p<0.01, ***p<0.001 compared to the control group).

5.3.2 The Rete Testis

Description of the rete testes of control and neonatally oestrogen treated rats will be presented in chronological order i.e. day 10 (neonatal), followed by day 18 (peripubertal), day 25 (pubertal), day 35 (late puberty) and day 75 (early adulthood).

Figure 5.2 panels (a, b) depict the rete testis of a control rat on postnatal day 10. Panels (a, c, e, f) show low power images, while panels (b, d, f, i) are high power images which allow the rete epithelium cells to be clearly viewed. Panel (a) demonstrates the position of the rete testis at the apical pole of the testis just beneath the spermatic cord. The rete testis is clearly patent at day 10. Figure 5.2 (b) illustrates the features of the rete testis epithelium at this age. In control animals the rete testis epithelium was highly columnar/cuboidal in appearance and generally displayed large ovoid nuclei located towards the base of the cell.

Panels (c, d) demonstrate the effect of neonatal DES administration (10μg/injection). The animals were treated on alternate days between day 2-8 postnatal and killed on day 10, so these animals were assessed during the treatment period. Panel (c) illustrates that the rete has

accumulated fluid and undergone considerable distension and appears to have partially invaded the testicular parenchyma. The irregular shaped canaliculi of the rete testis in control animals has been replaced with regular almost sphere-like region, which appear stretched and turgid. Panel (d) highlights that the distension has been accompanied by a change in the rete testis epithelium. The high columnar cells, which should characterise the epithelium at this age, appear low cuboidal/squamous in appearance.

Panels (e, f) and (g, h) show the rete testis from rats treated with bisphenol A (a weak 'environmental' oestrogen) and genistein (a phytoestrogen) respectively. Neither of these compounds produced any noticeable effect on rete testis morphology and, at both low and high power, the rete testis appeared comparable in size and morphology to that in the control animal shown in panels (a, b).

Figure 5.3 illustrates the rete testis at day 18 postnatal, which is around the time that seminiferous tubule fluid production is increased, Sertoli cell junctions are formed in the testis and lumens are formed in the seminiferous tubules. Panel (a) depicts the rete testis in a control animal at day 18. It is clear that the epithelial cell height is reduced compared to day 10 and lumens are evident within the testicular cords. Panel (b) shows the rete testis after neonatal administration of a GnRHa. It is clear that the rete testis appears less distended than the control (panel a) and still maintains the epithelial cell pattern characteristic of the day 10 rete. Lumens have not yet developed within the seminiferous cords. Overall these animals appear less developed than the corresponding controls.

Figure 5.3 (c, d) demonstrates the effect of neonatal ethinyl oestradiol and DES administration respectively (both 10μg/injection). Both treatments induced rete testis distension but it was evident that DES invoked a more severe and invasive distension than did ethinyl oestradiol. DES administration caused the rete testis to invade into the testicular parenchyma such that the rete testis no longer occupied only the superficial region underneath the testicular capsule. It was also quite evident that the testicular cords were not as well developed as in control animals with few cords, if any, showing signs of lumen formation. Panels (e, f) illustrate the effect of administration of lower doses of DES (1 and 0.1μg/injection, respectively). Administration of 1μg/injection induced a level of rete testis distension, which was similar in severity to that observed after the administration of ethinyl oestradiol (10μg/injection). The administration of DES appeared to cause the rete testis to invade further into the testicular parenchyma than after treatment with ethinyl oestradiol. Testicular development appeared impaired in both ethinyl oestradiol- and DES (10μg) – treated groups when compared to controls (panel a), as few seminiferous tubules showed lumen formation. At the lower dose of DES (0.1μg/injection) there was no apparent distension of the rete testis and its morphology and

that of the testis appeared similar to control animals with seminiferous tubules displaying lumen formation. Figure 5.3 (g, h, i, j) illustrates the effect of neonatal exposure to weak oestrogens (octylphenol, bisphenol A, parabens and genistein, respectively). None of these treatments induced any obvious rete testis distension and both rete testis and testicular morphology was similar to the control group.

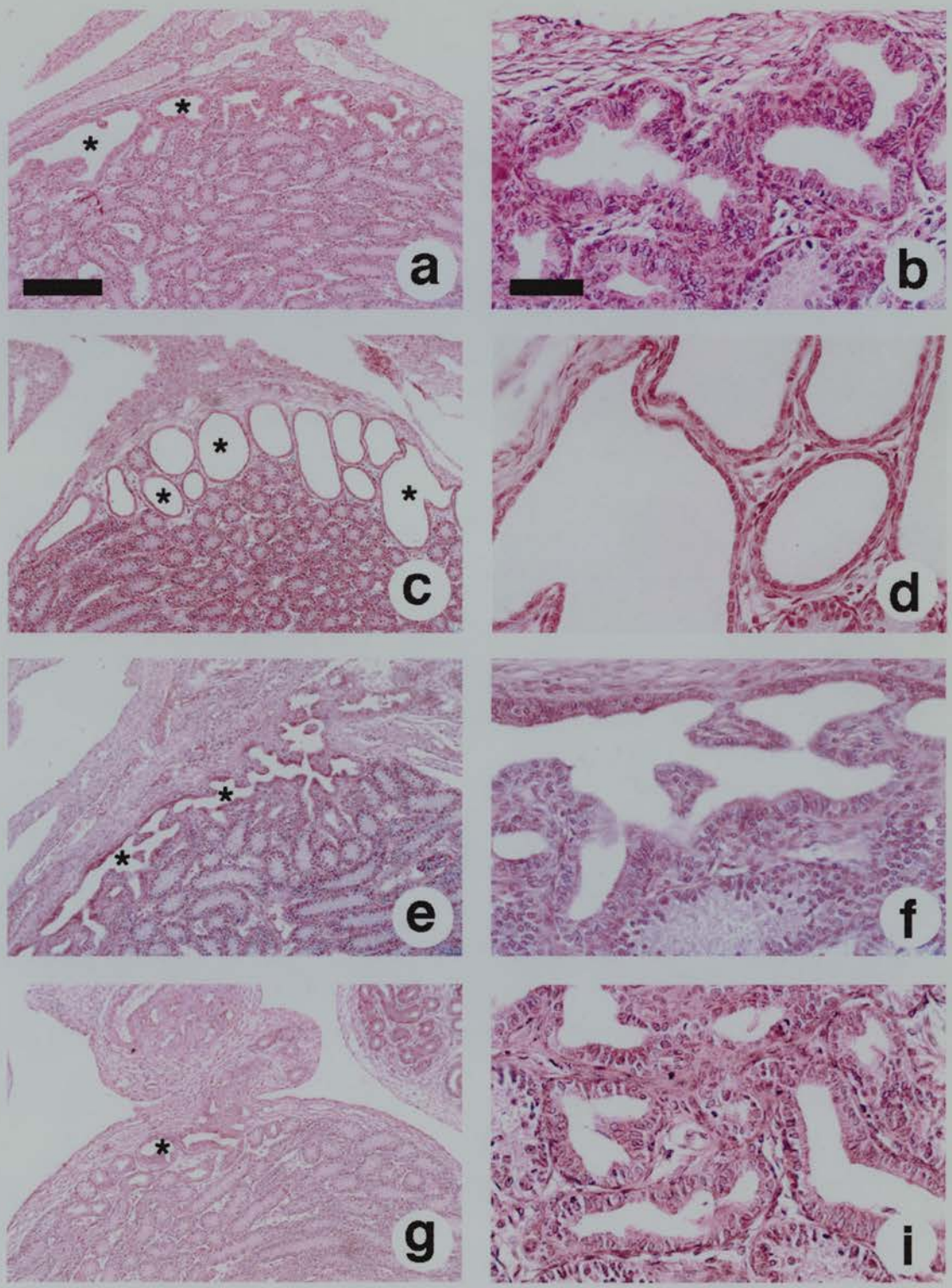

Figure 5.2 The Morphology of the Rete Testis in Control and Treated Animals at Day 10
Morphology of the rete testis (shown by asterisks) at 10 days of age in control rats (a, b) and in animals treated with DES (10μg/injection; c, d), bisphenol A (e, f) or genistein (g, h). panels a, c, e and g are low power images and the scale bar in (a) denotes 200μm. The images shown in panels b, d, f and h are high power and the scale bar measures 50μm.

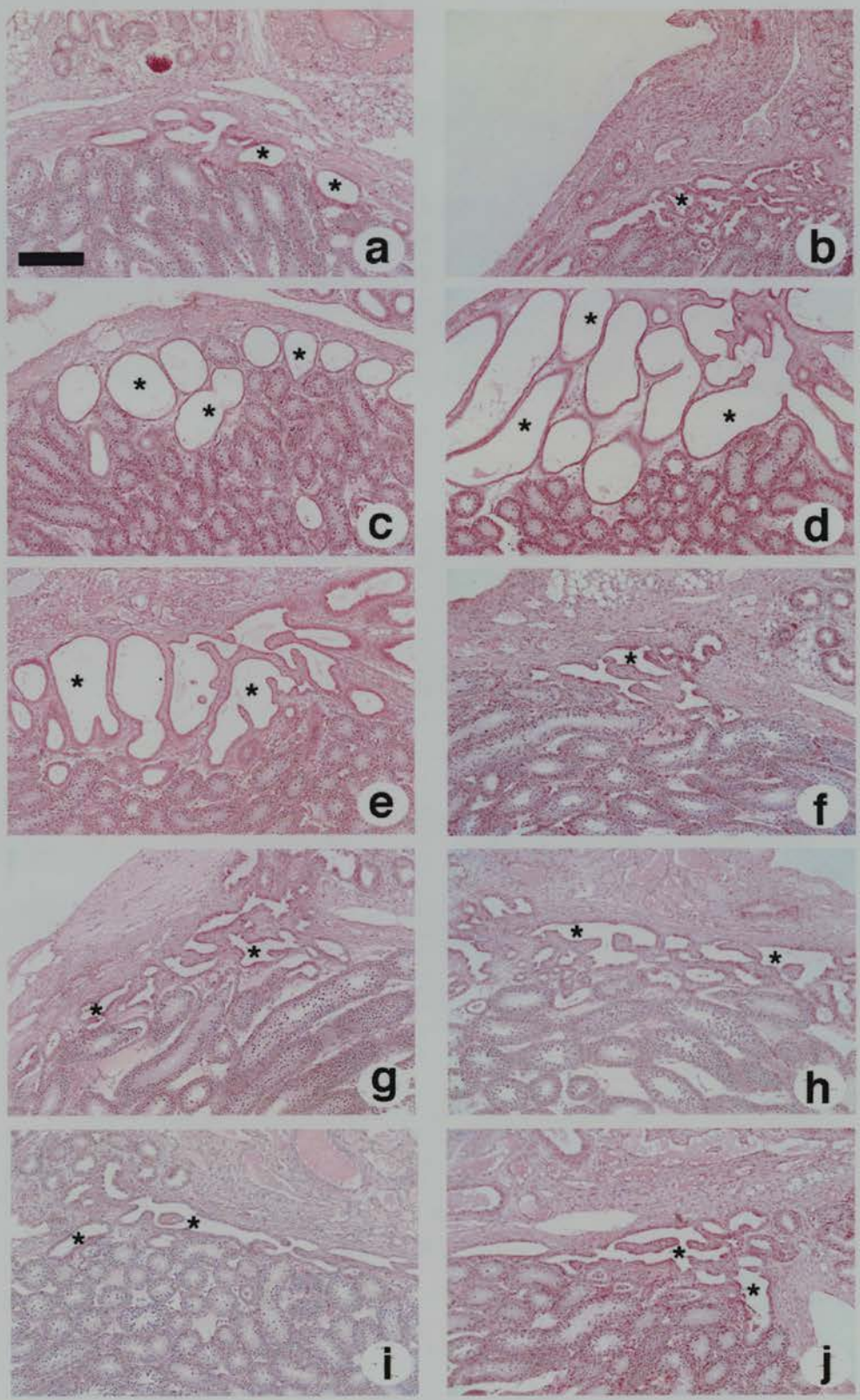

Figure 5.3 The Morphology of the Rete Testis in Control and Treated Animals at Day 18
Morphology of the rete testis (shown by asterisks) in rats aged 18 days. Panels show the rete testis from rats treated with (a) vehicle control, (b) GnRHa, (c) ethinyl oestradiol (10μg), (d, e, f) DES at 10, 1.0 and 0.1μg/ injection respectively, (g) octylphenol, (h) bisphenol A, (i) parabens or (j) genistein. The scale bar (a) denotes 200μm.

At day 25 (Figure 5.4) the control rete testis appeared more distended than at day 18, presumably reflecting the increase in STF production at puberty. Panel (b) illustrates the rete testis after neonatal exposure to GnRHa that appeared similar to control animals although the rete testis epithelium appeared higher and more cuboidal than in control animals. Panel (c) illustrates the rete testis after neonatal treatment with ethinyl oestradiol. There was no obvious sign of rete testis distension. The only animals which showed severe distension of the rete testis at day 25 postnatal were those which were treated with 10μg DES, a change still evident almost two weeks after the cessation of DES administration. Treatment with lower doses of DES (1 and 0.1μg/ injection; panels e, f, respectively) did not result in any signs of rete testis distension and appeared similar to control animals. Similarly, there was no evidence of rete testis distension after postnatal treatment with octylphenol (panel g), bisphenol A (panel h) or genistein (panel, i).

Figure 5.5 illustrates the rete testis at day 35. At this age, there was no obvious difference in morphology of the rete testis in control or GnRHa treated animals (panel a, b respectively). In contrast panels (c) and (d) depict rete testes from DES treated animals (10μg and 1μg/injection respectively). In comparison with the control (panel a) both were noticeably distended. Panel (c) (DES (10μg/injection) demonstrated an abnormal sloughing of germ cells into the lumen of the rete testis (compared with control (panel (a)), suggesting that spermatogenesis was adversely affected. Animals treated neonatally with ethinyl oestradiol were not assessed at this time point; those treated neonatally with DES at 0.1μg/injection (panel e) or bisphenol A (panel f) appeared similar to control animals.

The rete testis of rats treated neonatally with oestrogenic compounds was also assessed in early adulthood (day 75, Figure 5.6). The rete testis of control and GnRHa treated animals appeared similar with no signs of abnormal rete testis distension (panels a, b). However, animals treated neonatally with either ethinyl oestradiol or DES (10μg/injection) still showed signs of rete testis distension, although not as severe as that observed at day 18. Panels (d, g) illustrate the effect on testis morphology after DES (10μg/injection) but comparable results were obtained after treatment with ethinyl oestradiol. There were a large number of seminiferous tubules, particularly in the region of the testis directly underneath the rete testis, which were largely devoid of germ cells or were Sertoli-cell- only tubules. The rete testis from animals treated neonatally with lower doses of DES (1.0 or 0.1μg) or bisphenol A did not show any signs of distension and appeared similar to control animals.

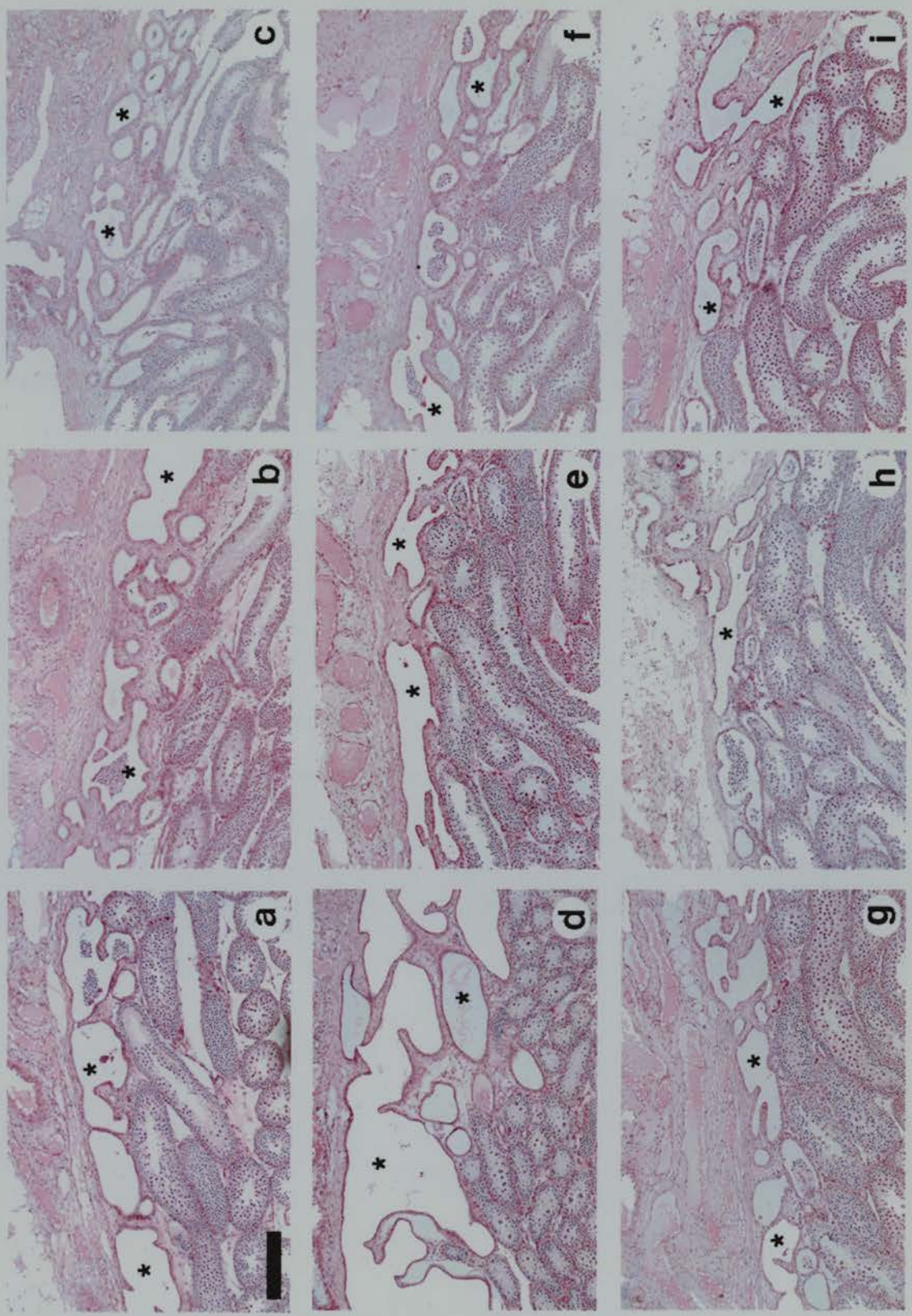

Figure 5.4 The Morphology of the Rete Testis in Control and Treated Animals at Day 25
Morphology of the rete testis in rats aged 25 days (shown by asterisks). panels show the rete testis from rats treated with (a) control vehicle, (b) GnRHa, (c) ethinyl oestradiol (10μg), (d, e, f) DES at 10, 1.0 and 0.1μg/injection respectively, (g) octylphenol, (h) bisphenol A, or (i) genistein. The scale bar (a) denotes 200μm.

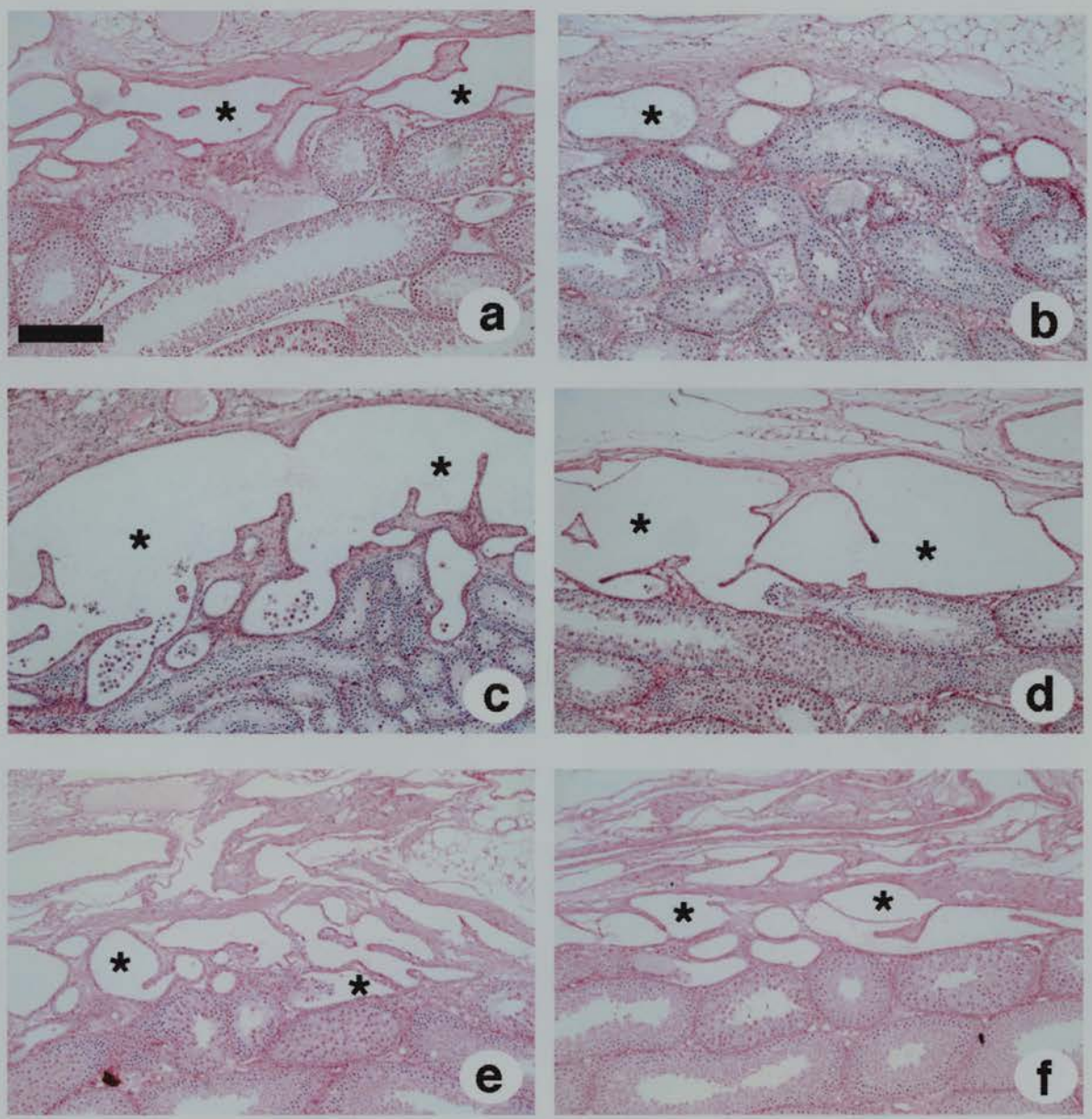

Figure 5.5 The Morphology of the Rete Testis in Control and Treated Animals at Day 35
Morphology of the rete testis in rats aged 35 days (shown by asterisks). Panels show the rete testis from rats treated with (a) control vehicle, (b) GnRHa, (c, d, e) DES at 10, 1.0 and 0.1μg/ injection respectively or bisphenol A (f). The scale bar (a) denotes 200μm.

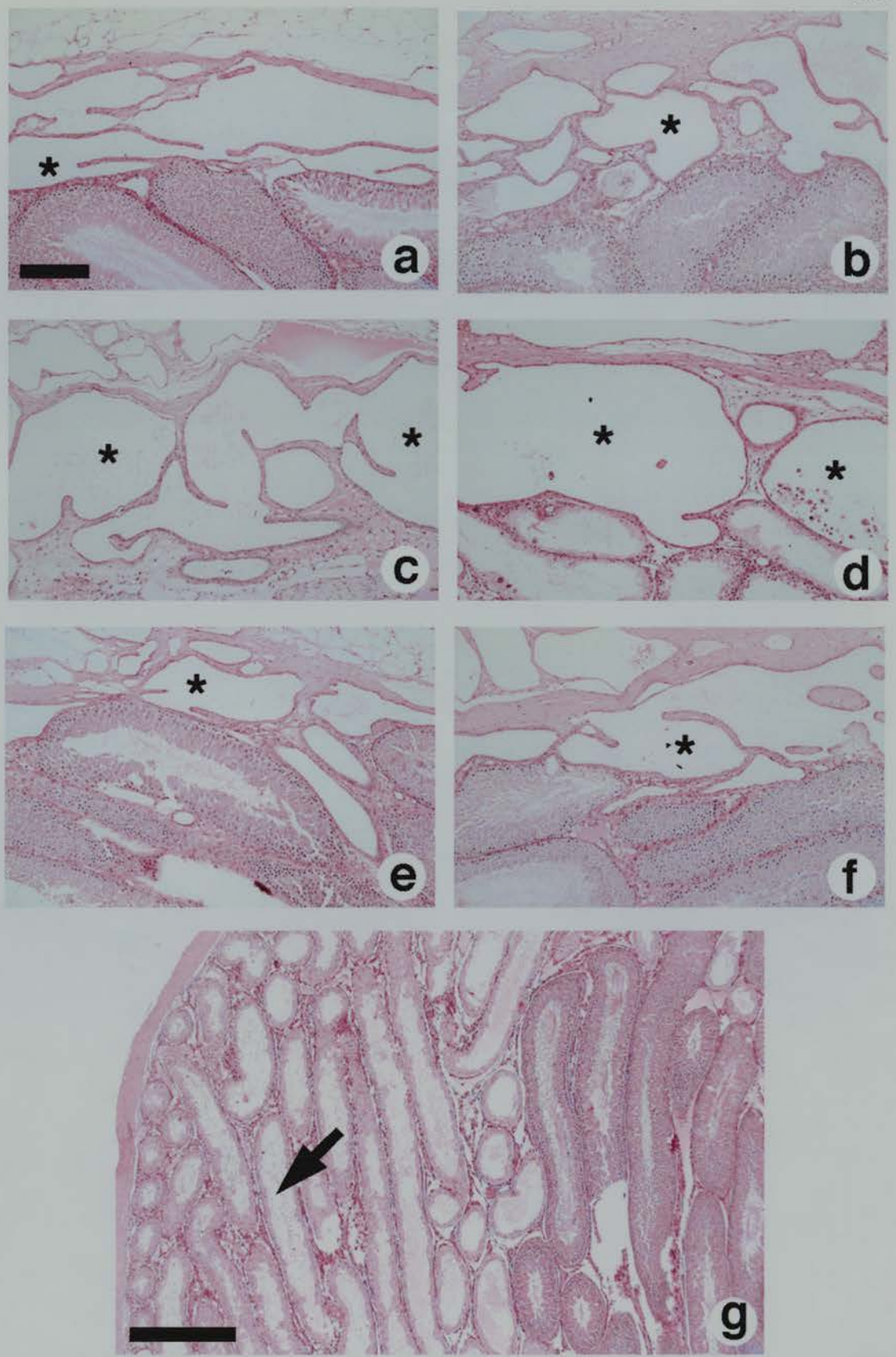

Figure 5.6 The Morphology of the Rete Testis in Control and Treated Animals at Day 75
Morphology of the rete testis in rats aged 75 days (shown by asterisks). Panels show the rete testis from rats treated with (a) control vehicle, (b) GnRHa, (c) ethinyl oestradiol (10μg), (d, e) DES at 10, and 0.1μg/injection respectively or bisphenol A (f). The scale bar (a) denotes 200μm. panel (g) shows a cross section from an adult rat after neonatal DES (10μg/injection) treatment. Scale bar shows 500μm and the arrow (g) illustate the region of disrupted spermatogenesis.

5.4 Discussion

Testis weight is a parameter that is often assessed in studies to determine toxic insult to the reproductive tract. In this study, the effect of various oestrogenic compounds (both weak and potent) on testis weight was determined and are shown in Figures 5.1 A-C. These figures show the overall increase in testis weight during postnatal development (day 18) until adulthood (day 75). On day 18 the mean testis weight in controls was 64.3±12.1mg and this had increased to 1989.9±175.0mg by day 75. Suppression of the secretion of gonadotrophins from the pituitary gland (using a GnRH antagonist) induced a permanent reduction in testis weight. This was apparent at all time points examined. By day 75 (Figure 5.1C), the testis weight in GnRHa treated animals was approximately 50% of control, due to a reduction in Sertoli cell numbers (Atanassova *et al.*, 1999), but spermatogenesis was grossly normal.

In rats treated neonatally with DES (all doses) there was a permanent reduction in testis weight in adulthood. This reduction was mirrored by administration of ethinyl oestradiol at least up to day 35 (data not shown). As with GnRHa, all of these treatments induce a permanent, dose-dependent reduction in Sertoli cell numbers (Atanassova *et al.*, 1999). At day 18, neonatal administration of tamoxifen had also decreased testis weight to levels comparable with GnRHa. The high degree of reduction induced by these chemicals (GnRHa, DES, ethinyl oestradiol and tamoxifen) suggests that they may all be capable, to some degree or other, of inhibiting the hypothalamo-pituitary axis. Studies have shown that suppression of this axis induces a reduction in testis weight (Blanco-Rodriguez and Martinez-Garcia, 1996).

While octylphenol initially appeared to increase testis weight (Figure 5.1A), by day 25 this was no longer apparent. The reason for the initial increase is not known. At day 18, neither parabens nor bisphenol A (at any age) had any significant effect on testis weight. At day 18 (Figure 5.1A), animals treated with genistein showed a decrease in testicular weight compared to controls maintained on a soy-free diet, but was not significantly altered from normal soy-fed controls. However, by day 75 (Figure 5.1C) testis weights of the genistein treated cohort were significantly increased when compared to normal control animals. The reasons for this are unknown. Weakly oestrogenic compounds did not appear to inhibit normal testis growth using the treatment protocols assessed in these studies. However, more potent compounds (i.e. more oestrogenic at the same dose) appeared to have a detrimental effect on testicular development as assessed by their effects on testis weight.

The literature review discussed the idea that in adults the bulk of testis weight is due to the germ cells and that this number is determined ultimately by the number of Sertoli cells.

Sertoli cell mitosis in the rat occurs prior to puberty and is partly an FSH driven process. Therefore any reduction in pituitary function (via GnRHa or oestrogen administration) leading to a reduction in FSH levels during this critical time period may permanently disrupt testicular development. In prepubertal animals (day 18), around the time the initiation of spermatogenesis, exposure to potent oestrogens (DES, ethinyl oestradiol) and GnRHa induced highly significant reductions in testis weight. As the treatments were administered during early neonatal life (i.e. while Sertoli cell replication was occurring) it is possible that the number of Sertoli cell mitoses has been reduced (and consequently the number of germ cells), in those animals showing a reduction in testis weight. Alternatively, these treatments may affect the replication of germ cells, or at later ages the ability of Sertoli cells to nurture the developing spermatozoa. Recent studies have shown that both Sertoli cell number and germ cell/per Sertoli cell are significantly reduced after neonatal treatment with ethinyl oestradiol (10μg) demonstrating a reduced efficiency of Sertoli cell support for spermatogenesis (Atanassova *et al.*, 1999). These authors also detected increased numbers of apoptotic germ cells after both DES and ethinyl oestradiol treatment which occurred across the spermatogenic cycle (Atanassova *et al.*, 1999).

The studies reported in this chapter have not attempted to investigate the effects of neonatal oestrogen exposure on the testis. It is well known that neonatal exposure to potent oestrogens during gestation and early neonatal life induces permanent disruption to spermatogenesis which are believed to involve both direct and hypothalmo-pituitary mediated effects (Atanassova *et al.*, 1999). Although the exact mechanisms remain unclear, direct effects are believed to include oestrogen effects on the regulation of Sertoli cell cytoskeletal reorganisation along the spermatogenic cycle (Blanco-Rodriguez and Martinez-Garcia, 1996). The cytoskeleton seemed a probable candidate as the germ cell sloughing observed after oestrogen treatment was not stage related. The loss of germ cells produces a tubule which appears to only have germ cells in the basal compartment, as the adluminal germ cells do not have enough time to mature before being sloughed into the seminiferous tubule lumen (Blanco-Rodriguez and Martinez-Garcia, 1996). Other studies, which have assessed spermatogenesis in animals treated using the same protocols described in this chapter, have found that DES induced dose-dependent reductions in germ cell and Sertoli cell volume per testis, and that Sertoli cell number is dose-dependently reduced at postnatal day 18 in rats (Atanassova *et al.*, 1999). The authors of the latter study suggest that changes in Sertoli cell function or altered fluid dynamics within the male excurrent duct system may be causal factors in the changes observed in spermatogenesis.

As part of the studies examined in this chapter, changes in rete testis morphology in response to oestrogenic compounds were examined (Figure 5.2-5.6). Profound changes occurred after

neonatal high dose oestrogen treatment (DES 10 and 1.0 μg and ethinyl oestradiol 10μg). At day 18, neonatal exposure to DES (10, 1, 0,1 μg/injection) induced dose-dependent distension of the rete testis with DES (10μg/injection) displaying the most severe effects. The rete testis of animals treated with DES (0.1μg/injection) showing no observable signs of fluid accumulation, whilst those treated with DES (1μg/injections) showed an intermediate level of distension.

The most severe distension of the rete testis in DES (10μg)-treated rats was evident at days 18 and 25 postnatal, whereafter the effects were less severe. The highest dose of DES however induced permanent distension as the rete testis was still swollen in comparison to control animals at day 75. How quickly after DES administration rete testis distension occurs was not assessed by this study but distension was evident in rats at day 10 postnatal (Figure 5.2) which was during the treatment period, and at this age the distension was less pronounced than at days 18 and 25.

In animals that were treated with ethinyl oestradiol (10μg), a comparable dose to the highest dose of DES administered, rete testis distension was also evident when assessed at day 18. The level of distension was not as severe in comparison with a similar dose of DES. However the induction of rete testis distension after both DES and ethinyl oestradiol treatments (both at 10μg) demonstrates that this effect is due to an oestrogenic effect and not to other toxic effects of DES. The distension was not caused by an oestrogenic effect on the hypothalamo-pituitary-gonadal axis as there was no sign of distension in the animals treated neonatally with GnRHa. Neonatal treatment with high doses of weakly oestrogenic compounds (octylphenol, bisphenol-A, parabens and genistein) did not induce any abnormal distension of the rete testis.

Abnormal distension and overgrowth of the rete testis has been reported in a few other studies which have analysed the effects of either gestational or neonatal treatment with DES or ethinyl oestradiol on male rodents. In a recent study a single injection of oestradiol benzoate (0.5mg/5g body weight or 1mg/ 5g bodyweight) was administered to rats on the day of birth (Aceitero *et al.*, 1998). In adulthood these rats showed disrupted spermatogenesis in a manner which bears a striking resemblance to Figure 5.6 (g) with the most pronounced degenerative changes in spermatogenesis found in the cranial region of the testis. Dilatation of the rete testis in these animals including both intra- and extra- testicular rete was observed from day 10. The degree of rete testis distension induced was of similar magnitude to the shown in Figure 5.6 (g) despite the massive dose of oestradiol benzoate administered by Aceitero *et al.*,(1998). This study also reported enlarged lumens in some seminiferous tubules near the dilated rete testis, even at day 10 postnatal, which the authors suggest

indicates a retrograde flow of fluid (Aceitero *et al.*, 1998). Distended seminiferous tubule lumens were not apparent in the work presented in this chapter (Figure 5.2-5.6) and therefore, this phenomena may only be induced after exposure to the high level of oestradiol benzoate used by Aceitero *et al.* This group also reported the presence of multinulcleate germ cells in the seminiferous epithelium and evidence of germ cell sloughing from day 22 postnatal. Evidence of germ cell sloughing was evident within the rete testis of our animals but this effect did not seem to be evident until the rats were 35 days of age (Figure 5.5 panel(c)) suggesting that the impairment of spermatogenesis occurred slightly later in our animals. However, this difference in timing could simply be a question of the dose of oestrogen administered.

The results described in this chapter demonstrate that DES produced gross distension and apparent overgrowth of the rete testis epithelium. The rete testis appeared to invade the testicular parenchyma instead of remaining in its superficial position under the tunica. This effect seems unlikely to be explained just by the distension. Abnormalities of the rete testis have been described in mice which were exposed during gestation to 100μg/Kg DES administered to the mother on days 9-16 of gestation and then examined in adulthood (Newbold *et al.*, 1985). A total of 233 DES-exposed male mice were examined and 56% of these displayed various degrees of rete testis hyperplasia which consisted of knob-like protrusions of cuboidal epithelia or proliferative overgrowths forming papillary structures consisting of small cuboidal cells. These structures were never found in control animals. Of the DES treated males, 5% displayed a severe adenocarcinoma-like lesion that often infiltrated into the seminiferous tubules or efferent ducts. Rete testis adenocarcinoma is a very rare neoplasm in both humans and animals and based on the results of Newbold's experiments, prenatal exposure to DES induced a 20-fold increase in the prevalence of rete adenocarcinoma-like lesions (Newbold *et al.*, 1985; Newbold *et al.*, 1986).

The reason why neonatal oestrogen administration induces distension and the apparent accumulation of fluid is not clear, and this is particularly true in animals assessed at day 10 postnatal before the Sertoli cell tight junctions and seminiferous tubule lumens have formed. The accumulation of fluid in the rete testis at day 10 postnatal suggests that fluid is flowing through the male reproductive tract prior to the formation of seminiferous tubule lumens and Sertoli cell junctions or alternatively, that fluid may enter the rete testis from another source such as lymphatic fluid. It is known that the rete testis can reabsorb fluid so it may be possible that the epithelium could undergo a 'polarity reversal' and become secretory. Oestrogen can induce mammary epithelial cells in culture to undergo a reversible loss of polarity (Failka *et al.*, 1996). Oestrogen, acting via a c-JunER fusion protein, induced a transcriptional regulation of a variety of AP-1 target genes. The loss of epithelial cell polarity involved the redistribution of both apical and basolateral proteins to the entire plasma

membrane (Failka *et al.*, 1996). If such a reversal of cell polarity could be induced in the rete testis *in vivo,* it might result in an epithelium which secretes fluid instead of resorbing it.

Based on these results the induction of rete testis distension only occurs after the administration of potent oestrogens and the severity of the distension varies dose-dependently. However, as distension was never induced after exposure to weakly oestrogenic compounds it is concluded that rete testis distension is not a useful marker to assess exposure to 'environmental oestrogens'. The available evidence suggests that exposure to inappropriate levels of oestrogen can induce perturbations in normal fluid dynamics within the excurrent duct system of the male reproductive tract and these changes could have consequences for male fertility. As the major site of fluid resorption within the male reproductive tract are the efferent ducts, the effect of neonatal oestrogen exposure on this tissue will be addressed in Chapter 6.

Chapter 6 Morphological Changes in the Efferent Ducts After Neonatal Oestrogen Treatment

6.1 Introduction

Chapter 5 assessed the effects of neonatal oestrogen exposure on the morphology of the rete testis. This chapter initially assessed the morphological changes induced in the efferent ducts of the epididymis by neonatal exposure to oestrogenic compounds and then assessed changes in the expression of functional markers such as Aquaporin-1 and the androgen and oestrogen steroid receptors at pertinent time-points.

An extensive introduction to the morphology of the efferent duct epithelium and its role in fluid resorption has been described in Chapter 1 (Section 1.3.2) and will not be repeated here. Similarly, the rationale and basic structure of the treatments administered in these studies was described in the Introduction to Chapter 5 (Section 5.1) and the reader is referred to these sections. The efferent ducts of the epididymis share one striking similarity to the rete testis, in that together they comprise the least studied structures in male reproductive physiology! This is because they are regions that are largely destroyed when the testis is dissected from the epididymis by researchers studying one or other of these two organs. This introduction will address what is known regarding the hormonal control of the efferent ducts and the effects that have been induced by toxic insult to this tissue.

In general, the hormonal control of the excurrent ducts is ascribed to androgens (which is largely true for the epididymis), but the epithelia of the rete testis, efferent ducts and initial segment of the epididymis also appear to be under the control of other testicular factors present in luminal fluid (Klinefelter and Hess, 1998). The exact mechanisms involved in the regulation of the efferent ducts are not understood. The effects of efferent duct ligation and androgen withdrawal have been addressed with respect to changes in the efferent duct epithelium. Fawcett and Hoffner found that efferent duct ligation in the rat at the level of the rete had little effect on the distal segment of the efferent ducts, but it did induce epithelial regression in the initial segment of the epididymis. This could not be prevented by the administration of exogenous androgens (Fawcett and Hoffer, 1979). Recent studies have found that rete testis ligation reduces the number of dense granules within the efferent duct epithelium (Klinefelter and Hess, 1998).

The activity of the Na^+/K^+-ATPase localised to the basal region of the efferent duct epithelium has been suggested to be stimulated by circulating androgens (Ilio and Hess, 1994) and

factors within the luminal fluid are important in modulating the enzymatic activity of the epithelium. Castration or ligation of the ducts near the testis decreases enzyme activity in both the efferent ducts and initial segment of the epididymis; this could not be completely restored by exogenous androgen (testosterone or dihydrotestosterone) administration. The administration of 17β-oestradiol to intact rats lowered enzyme activity in both the proximal segments of the efferent ducts and caput epididymis but did not affect the distal conus region of the efferent ducts or the initial region of the epididymis (Ilio and Hess, 1994). This suggests that oestrogens may selectively modulate the activity of certain enzymes in different regions of the excurrent ducts.

Chapter 3 of this thesis and the published literature described in Chapter 1 (section 1.7.1 and 1.7.4) demonstrate the known expression of the AR and ERs (α and β) within the efferent duct epithelium. The expression of these sex steroid receptors suggests that the efferent ducts are a target for sex steroid hormone action. However, the functional significance of their expression remains to de determined. Hormone receptors for opioids and proenkephalin have also been reported in the efferent duct epithelium but their functions are not known (Klinefelter and Hess, 1998).

A major problem in trying to identify the direct effects of oestrogen on the excurrent ducts is that oestrogen is known to have inhibitory effects on both the testis and pituitary gland. The only way to decipher cause and effect in these studies was to compare the effects induced after DES administration with those induced when the reproductive axis was shut down by the administration of a GnRHa. There have been many studies which have examined the effect of hypophysectomy (removal of the pituitary gland) or oestrogen administration as a means of disrupting spermatogenesis (Blanco-Rodriguez and Martinez-Garcia, 1996). Both techniques induce a marked decrease in testicular weight, in seminiferous tubule diameter and the number of germ cells within the seminiferous epithelium, together with a thickening of the basement membrane (all features observed with the current DES and ethinyl oestradiol (10μg/injection)). Hypophysectomy also causes a major decline in the levels of plasma FSH, LH and testosterone, and similar but more gradual decreases have been described after oestrogen treatment (Blanco-Rodriguez and Martinez-Garcia, 1996). As these effects are also shared by animals treated with GnRHa, they are indirect actions of oestrogens and so will not be pursued by the present studies as it is the aim of this thesis to determine the direct effects of oestrogen induced on the excurrent duct system.

Studying the toxic effects of chemicals, specifically on the excurrent ducts of the male is a relatively new field as it shares the problem of trying to separate direct and indirect effects, as just discussed. This is because many of the chemicals administered to animals affect testicular function and the functional impairment of Sertoli or Leydig cells can have pervasive influences on the functioning of the excurrent duct epithelia. Aside from a major role in fluid resorption, the efferent ducts are also involved in sperm and ion transport, protein resorption, steroid metabolism and spermiophagy (Ilio and Hess, 1994). These processes can be affected by the administration of toxic compounds that then have specific effects on the efferent ducts.

α-Chlorohydrin (3-chloro-1,2-propanediol) is a chemical which causes damage within the male reproductive tract, particularly the efferent ducts. At high doses it induces histopathological lesions in the efferent ducts and initial segment of the epididymis and at lower doses it has direct effects on sperm, (Mohri *et al.*, 1975). High doses of α-chlorohydrin induce the formation of intratubular spermatoceles in the efferent ducts which are more severe towards the rete testis (Cooper and Jackson, 1972). The epithelium of the spermatoceles is fragmented and sperm can be seen within the underlying connective tissue resulting in granuloma formation and ultimately hyalinization and ductal fibrosis. The first effects of exposure are ductal occlusion, sperm stagnation and subsequent luminal dilatation. The dilation of the efferent duct lumen is thought to induce an inflammatory response and sperm granuloma formation. The lack of fluid movement causes the rete testis to become swollen with seminiferous tubule fluid which can flow back due to increased pressure which can lead to atrophy of the testis (Ilio and Hess, 1994). Ethane dimethanesulphonate (EDS) which is used to ablate Leydig cells, also induces hyperplasia of the efferent ducts and small intratubular sperm retention cysts have been observed (Cooper and Jackson, 1972). Five to seven days after EDS treatment these cysts join into large spermatoceles which can rupture and form granulomas (Cooper and Jackson, 1973). Unlike α-chlorohydrin, EDS lesions can be resolved and fertility recovers in some males (Cooper and Jackson, 1973).

Exposure to environmental fungicides (benzimidazole carbonates) such as benomyl and its metabolite carbendazim induce occlusion of the efferent ducts (Klinefelter and Hess, 1998). These chemicals are used for controlling mould, fungus and other organisms that infest lawns, gardens and orchard plants. Within hours of administration, benomyl induces increases in testicular weight and in the long-term this leads to testicular atrophy and infertility. As with the other toxins described above, benzimidazole carbonates induce occlusion of the efferent ducts, and fluid back-pressure then causes seminiferous tubule swelling, loss of germ cells and eventual testicular atrophy (Klinefelter and Hess, 1998).

Some chemicals that have toxic effects on the reproductive system have their primary effect elsewhere in the body. For example, effects on the reproductive tract can be induced by chemicals which affect the testicular vasculature. A 5-hydroxytryptamine-1 receptor antagonist (GR40370D, Glaxo Wellcome) was under investigation as treatment for migraine relief until it was found to have both testicular and epididymal toxicity. GR40370D was found to act on the testicular vasculature to induce constriction and in some cases closure of the veins of the mediastinal venous plexus and arterial-venous anastamoses respectively (Piner, 1997). Acute administration of GR40370D to adult rats caused an increase in testicular weight, increased diameter of seminiferous tubules and distension of the rete testis observed 12- 48h after administration. Within the epididymis, increased luminal size of the initial segment and caput regions, oedema of the interstitial space and an increase in organ weight were noted 24-48h post-treatment. These changes were reversible after 168h (7 days). The initial action of this drug was to induce vascular constriction and the mechanisms which led to oedema and reduced fluid resorption through the testis and excurrent duct system are unknown (Piner, 1997).

Exactly how all of these chemicals act to induce efferent duct lesions is not understood and there are few studies that have examined the morphology of the efferent duct epithelium after toxic injury. Toxicological studies examine doses and responses and there are very few studies, which examine mechanisms. This chapter aims to assess changes in the efferent ductules and their epithelium after exposure to both potent and weakly oestrogenic compounds and suggests possible mechanisms behind these effects.

6.2 Materials and Methods

6.2.1 Animals

The animals were housed and neonatal treatments administered as described in sections 2.1 and 2.2. To obtain sections of efferent ducts, tissue (which had been embedded in paraffin wax as described in sections 2.3.3 and 2.3.4) was carefully sectioned, examining every 5-10th paraffin section using light microscopy to locate the efferent ducts. When the efferent ducts were located the quality of the cross section was determined by haematoxylin and eosin staining (as described in Chapter 5, section 5.2.3).

6.2.1.1 Acute Administration of 5HT-1 Receptor Antagonist (GR40370D)

Adult rats were administered 75mg GR40370D/ml sterile water by oral gavage. The test solution was freshly prepared and administered within 4 hours at a volume of 10ml/Kg and the animals were killed 4 or 21 hours after dosing.

6.2.2 Image Analysis - Measurement of Epithelial Cell Height

H&E staining was performed on efferent duct cross-sections of all animals in all treatment groups at all time points examined in order to analyse the morphology of the efferent ducts after treatment. The staining protocol was performed as described in Chapter 5 (section 5.2.3). The slides were used to assess changes in efferent duct epithelial cell height. Epithelial cell height was measured using an Olympus BH2 microscope fitted with a 40x plan achromat objective and a 3.3x phototube (Olympus Optical). The image was captured using a Sony XC77CE video camera (Sony, Tokyo, Japan) linked to a personal computer with frame grabber and image pro image analysis software (Media Cybernetics, Silver Spring, MD). The length tool was used to measure the height of the epithelial cells at right angles to the basement membrane. The cell was measured from the centre of the basal cell membrane and a line drawn from the basal to the apical membrane. After measuring the length, the angle of the line was measured to ensure that it was 90 degrees. For each animal, at least 50 cells were measured with sampling from at least 3 ductule cross sections. The mean epithelial cell height was calculated for each animal. The data was assessed using analysis of variance and where significant differences were found, subgroup comparisons were analysed using the same test, but using the overall variance as the measure of error.

6.2.3 Immunocytochemistry on Paraffin Sections

6.2.3.1 Aquaporin-1 Immunocytochemistry

Aquaporin-1 immunocytochemistry was performed as described in Chapter 2, section 2.4.1 using the antibody described in Chapter 4 (section 4.2.2). The level of immunostaining was assessed in the efferent ducts from animals at all ages (10, 18, 25, 35 and 75 days) and from all treatment groups. To ensure that the changes described in immunostaining were real and were not an artefact of different times when the staining procedures were performed, immunocytochemistry was always performed with a section of control and treated tissue on the same slide. Any changes in the level of immunostaining were due to changes in protein levels as the slides had been treated identically. Immunocytochemistry was performed at least 3 times, on tissue from each animal to ensure the reproducibility of the results.

6.2.3.3 Sex Steroid Receptor Immunocytochemistry

The immunocytochemistry protocols used were identical to those described in Chapter 4 (sections 3.2.3, 3.2.4, 3.2.5).

6.2.4 Photomicroscopy

The slides were photographed as described in Section 2.5.1.

6.2.5 Western Blot Analysis

Western blots were performed using 75μg of protein from adult efferent duct and kidney and efferent ducts from control and DES (10μg/injection) treated rats aged 25 days postnatal. The proteins were separated using a 12% acrylamide SDS-PAGE, blotted onto a PVDF membrane and probed with AQP-1 antiserum and pre-immune serum at 1:5000. The methods are fully described in Chapter 2 (sections 2.6-2.8) using ECL detection.

6.3 Results

As formerly discussed in Chapter 5 the most striking change in neonatal animals administered potent oestrogens were fluid retention and the subsequent swelling of the rete testis. Therefore, initially the morphology of the efferent ducts was assessed to determine whether distension was present in this tissue.

6.3.1 Epithelial Cell Morphology

Figure 6.1 illustrates a cross section of control (panel a) and ethinyl oestradiol treated animals (panel b) at day 18 postnatal for comparison. Panel (a) demonstrates the high columnar appearance of the efferent duct epithelial cells. The nonciliated cells have basely situated nuclei and an extensive brush border on the apical membrane, whereas the ciliated cells have centrally located nuclei and a ciliated apical surface membrane. The epithelium is surrounded by a layer of periductular myoid cells (1-2 cells thick) and by loose connective tissue and stromal cells. In contrast, in the ethinyl oestradiol treated animal (panel b) efferent duct morphology was greatly altered. The most noticeable change was the reduction in epithelial cell height. Both ciliated and nonciliated cells had lost or not developed the majority of their apical cytoplasm. The apical brush border was greatly reduced but clumps of cilia could still be identified. It was not possible to distinguish the two cell types based on nuclear morphology as both now lay just above the basement membrane. Cell nuclear morphology was greatly changed, as they were reduced in size and were short, elongated and flattened against the basal side of the cell; the nucleus now occupied the majority of the intracellular space. The periductular tissue also appeared altered, with an increase in the cell density surrounding the ductule and an increase in the amount of connective tissue. These gross changes in morphology were present throughout the length of the efferent ducts and extended into the epididymis (but the latter was not addressed in these studies).

6.3.2 Epithelial Cell Height

To determine whether changes in cell height were induced after exposure to potent oestrogens and to determine the degree of change in cell height, epithelial cell height was measured. The

graphs shown below demonstrate changes in epithelial cell height induced after neonatal exposure to either potent or weakly oestrogenic compounds. The data for each age group are illustrated in separate graphs.

The data shown in Figure 6.2A is the epithelial cell height measured in 10 day old neonates which were still undergoing treatment. At this age, cell height in control animals (12.9±1.8μm) was indistinguishable from animals being administered large doses of the weakly oestrogenic compounds bisphenol A and genistein. However, there was already a significant reduction in epithelial cell height in animals treated with DES (10μg/injection; 8.7±1.0μm).

At day 18 (Figure 6.2B) there were numerous treatment groups as this was the time point which had shown the most severe effects after neonatal treatment with potent oestrogens (DES or ethinyl oestradiol). At day 18, all treatments, except butyl parabens induced a significant reduction in efferent duct epithelial cell height but the severity of the reductions varied considerably. Administration of either DES or ethinyl oestradiol both at 10μg/injection induced similar, major reductions in epithelial cell height (DES 10μg 7.8±0.9μm; ethinyl oestradiol 6.7±1.3μm) with values reduced to less than 50% of control cell height (Control 16±1.3μm). Treatment with GnRHa and the two lower doses of DES (1 and 0.1μg/injection) also caused smaller, but significant reductions in cell height which were of similar magnitude (GnRHa 11.6±1.5μm; DES 1μg 12.1±2.8μm; and DES 0.1μg 12.7±1.1μm), whilst animals treated neonatally with tamoxifen showed a smaller decrease (13.1±0.8μm). Animals treated with genistein also showed a small reduction in epithelial cell height when compared to that measured in control animals whose mothers were fed a soy-free diet; there was no statistical difference in epithelial cell height between soy free and 'normal' control animals. In all other graphs, data for control and 'soy-free' control animals have been pooled. Small but significant changes were noted in epithelial cell height after neonatal treatment with weakly oestrogenic compounds (octylphenol (14.5±1.1μm), and bisphenol A (13.9±2.4μm)).

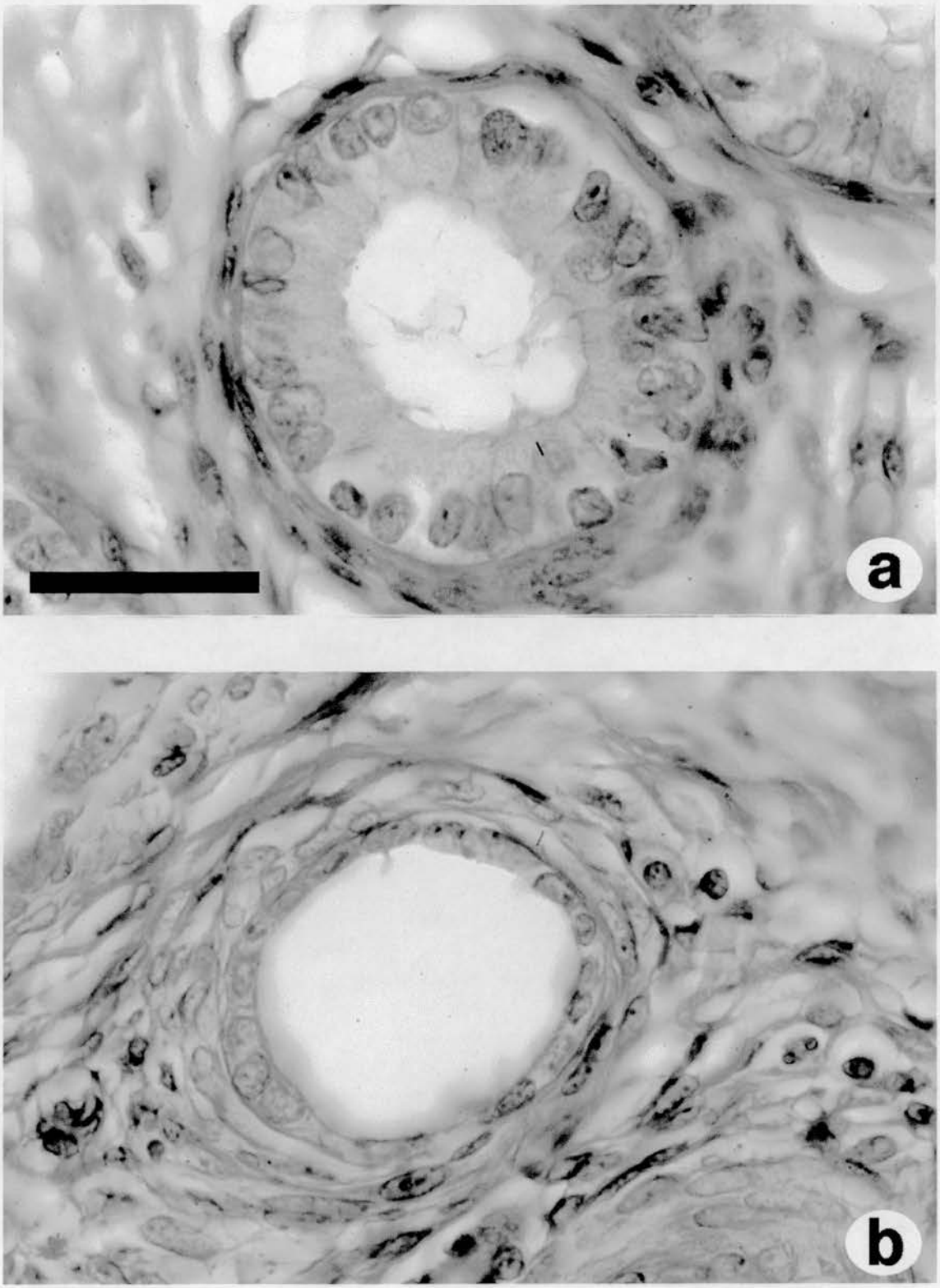

Figure 6.1 Comparison of Efferent Duct Morphology at day 18 in Control and Ethinyl Oestradiol Treated Animals

Photomicrograph of the efferent duct epithelium at day 18 in a control rat (a) and an animal treated neonatally with 10μg ethinyl oestradiol (b). Scale bar shows 20μm.

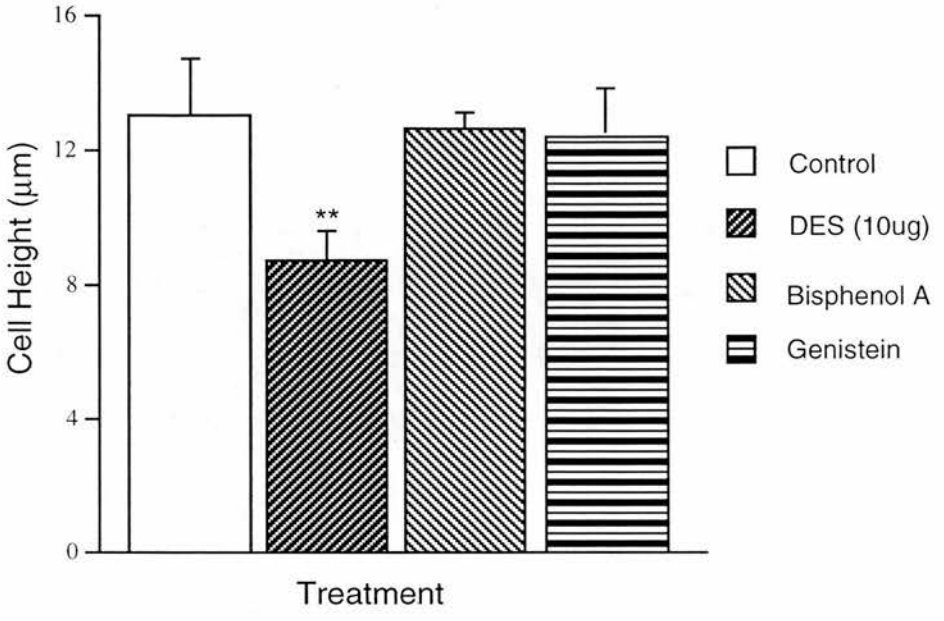

Figure 6.2A Measurement of Epithelial Cell Height (µm) at Postnatal Day 10

Animals were treated with a vehicle control (n=5), DES (10µg/injection; n=4), Bisphenol A (n=4) or genistein (n=5). Doses were administered as described in Section 2.2. **=p<0.01 compared to control animals.

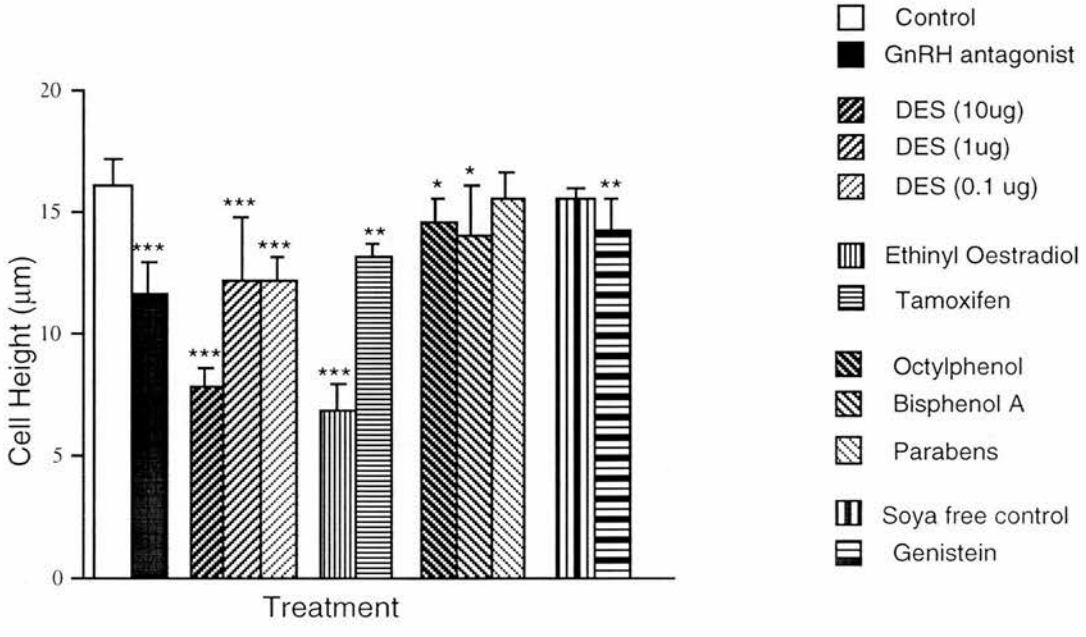

Figure 6.2B Measurement of Epithelial Cell Height (µm) at Postnatal Day 18

Animals were treated with a vehicle control (n=13), DES (10µg, 1µg or 0.1µg/injection; n=10, n=5, n=10 respectively), ethinyl oestradiol (n=4), tamoxifen (n=4), octylphenol (n=6), bisphenol A (n=5), parabens (n=5), soy free control (n=8) or genistein (n=7). Doses were administered as described in Section 2.2. (* p<0.05, ** p<0.01, *** p<0.001 when compared to controls).

Figure 6.2C shows the epithelial cell height data obtained from 25 day old animals. It is important to note that by day 25 there was no significant change in efferent duct epithelial cell height between control animals and those treated neonatally with GnRHa. Treatment with GnRHa at day 18 (fig. 6.2B) had induced a similar reduction to animals treated with lower doses of DES (1 and 0.1μg/injection), but the latter treatment groups still showed a significant reduction in epithelial cell height at 25 days of age, [DES 1μg, 12.5±4.1μm; DES 0.1μg, 10.5±1.9μm] when compared to controls (16.4±1.5μm). Higher doses of DES and ethinyl oestradiol (10μg/injection) induced highly significant reductions in epithelial cell height (DES 7.5±1.1μm; ethinyl oestradiol 7.2±1.2μm), with values approximately 50% of control levels. Neonatal treatment with either octylphenol (14.4±1.3μm) or bisphenol A (14.3±0.9μm) induced small but significant reductions in epithelial cell height. At 25 days of age, genistein treated animals no longer showed any significant reduction in epithelial cell height.

The epithelial cell height of neonatally oestrogen treated rats was also assessed at day 35 (Figure 6.2D). Similar to findings at day 25, animals treated with GnRHa displayed no difference in cell height when compared to control animals (Control =17.1±2.8μm, GnRHa 16.7±1.0μm). Treatment with the high dose (10μg) of DES still caused a highly significant reduction in cell height (11.9±2.3μm), but it was clear that this decrease was of smaller magnitude than that seen at day 25. Treatment with 1μg/injection DES still caused a significant reduction in cell height, whereas in animals treated with the lowest dose of DES (0.1μg/injection) cell height (13.7±2.7μm), was not significantly different from control animals (17.5±1.3μm). Similarly, animals treated neonatally with the weak oestrogen, bisphenol A showed no significant change in cell height at day 18 postnatal, whereas at day 25 of age this treatment had caused a small but significant decrease in epithelial cell height.

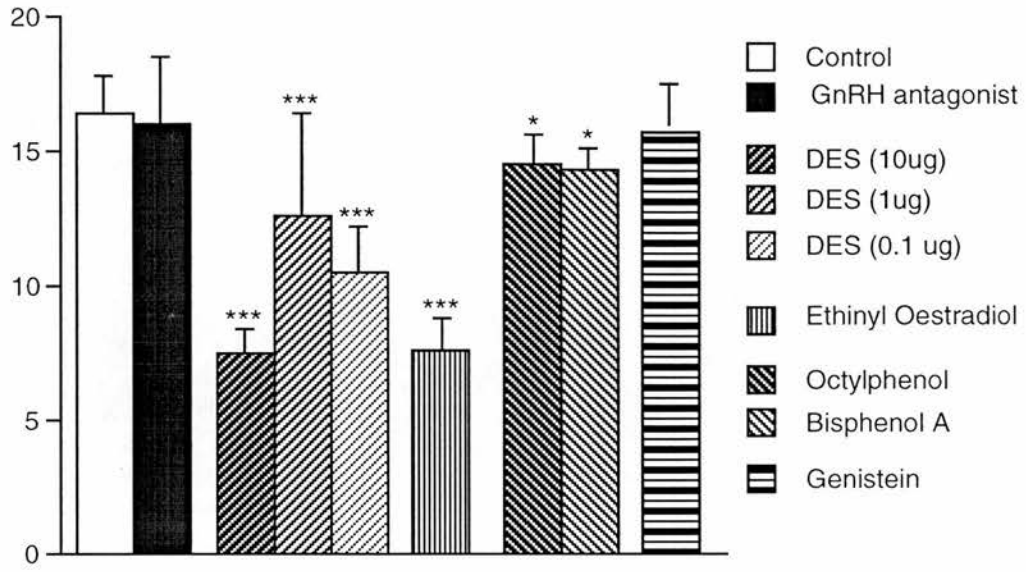

Figure 6.2C Measurement of Epithelial Cell Height (μm) at Postnatal Day 25

Animals were treated with a vehicle control (n=20), DES (10μg, 1μg or 0.1μg/injection, n= 9, n=4, n=3 respectively), ethinyl oestradiol (n=5), octylphenol (n=6), bisphenol A (n=4), or genistein (n=7). Doses were administered as described in Section 2.2. (* $p<0.05$, *** $p<0.001$ in comparison with controls).

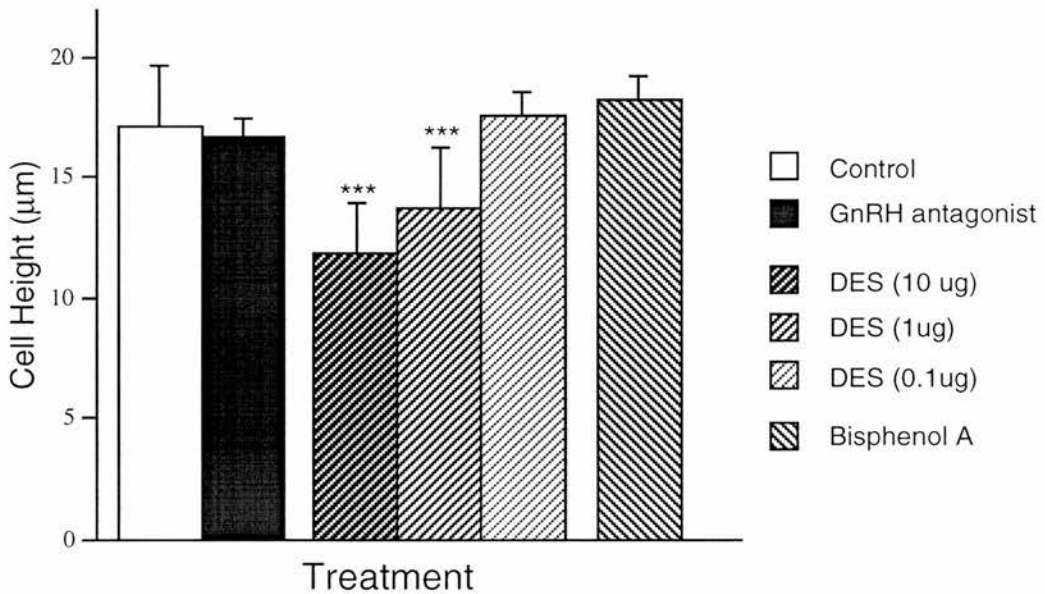

Figure 6.2D Measurement of Epithelial Cell Height (μm) at Postnatal Day 35

Animals were treated with a vehicle control (n=9), GnRHa (n=3), DES (10μg, 1μg or 0.1μg/injection; n=6, n=5, n=4 respectively), or bisphenol A (n=3). Doses were administered as described in Section 2.2. (*** $p<0.001$ in comparison with controls)

Cohorts of treated animals were allowed to mature into adults and the efferent duct epithelial cell height was assessed at day 75 (Figure 6.2E). At this age there was no significant change in epithelial cell height with any of the treatments assessed. There was a downward trend in epithelial cell height with increasing dose of DES but this did not reach significance.

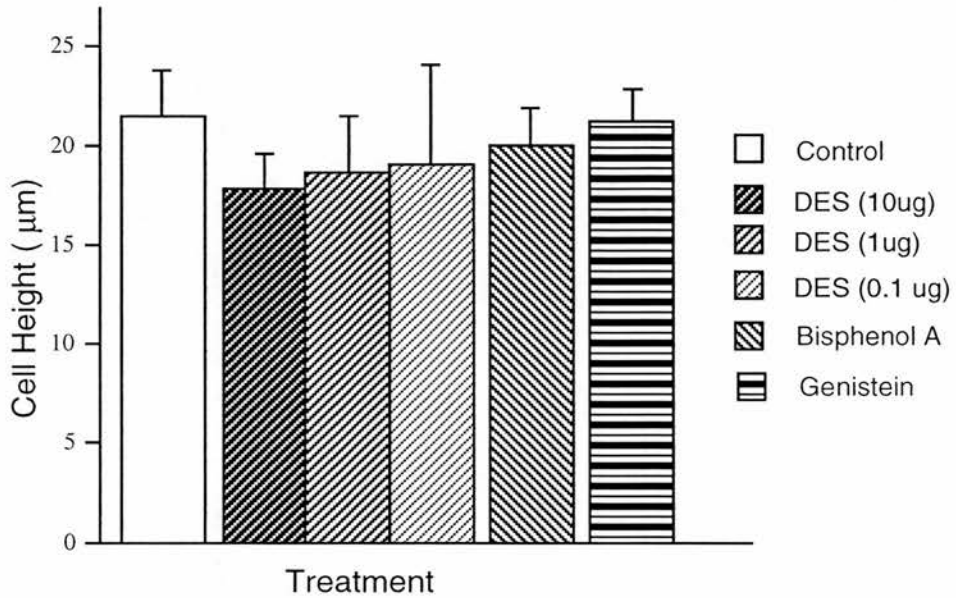

Figure 6.2E Measurement of Epithelial Cell Height (µm) at Postnatal Day 75

Animals were treated with either vehicle control (n=7), DES (10µg, 1µg or 0.1µg/injection; n=6, n=5, n=3 respectively), bisphenol A (n=3), or genistein (n=5). Doses were administered as described in Section 2.2.

6.3.3 Efferent Duct - Functional Changes

The previous section described the changes in efferent duct morphology induced by neonatal exposure to oestrogenic compounds. Given the gross morphological changes induced, this section addresses functional changes induced in the efferent ducts after neonatal exposure to potent or weak oestrogens.

6.3.3.1 Aquaporin-1 Immunoexpression After Neonatal Oestrogen Exposure

Aquaporin-1 immunocytochemistry was performed on tissue from all tissue from animals from all treatment groups at all of the time points sampled. Due to the volume of data this amassed, only selected ages and treatment groups will be presented but all the data has been tabulated and is shown in Table 6.1. Images from all treatment groups are shown for day 18 (Figure 6.3, 6.4, 6.5) but the data for day 25 has been omitted as the results were essentially identical to those at day 18. Selected data is shown for day 35 (Figure 6.6) and all data groups sampled at day 75 are shown (Figure 6.6).

At day 10 postnatal a cohort of control, DES (10μg/injection), genistein and bisphenol A treated animals were assessed for AQP-1 immunostaining (see Table 6.1). This was the only time point sampled when the animals were within the treatment period. In control animals, AQP-1 formed a strong apical band along the brush border of the efferent duct epithelial cells as shown in Chapter 4 (Figure 4.2). DES treatment (10μg/injection) greatly reduced the level of AQP-1 immunoexpression, the efferent duct lumens appeared distended, and epithelial cell height was noticeably reduced (Figure 6.1). Treatment with weakly oestrogenic compounds (genistein or bisphenol A) caused no detectable reduction in the intensity of AQP-1 immunostaining or changes in efferent duct morphology (see Table 6.1).

At postnatal day 18 (the results are summarised in Table 6.1), representative photomicrographs of efferent ducts at low (x400) and high power (x1000 oil immersion) are shown in Figures 6.3 and 6.4. A fluorescently stained photomicrograph is also shown in Figure 6.5 to display the level of residual AQP-1 immunostaining at 18 days of age after treatment with ethinyl oestradiol (10μg/injection). In control rats at day 18, AQP-1 immunostaining was clearly evident along the brush border of the efferent duct epithelial cells as shown in panel (a) Figure 6.3. The higher resolution photomicrograph in panel (b) clearly shows that AQP-1 immunostaining was evident along the lateral membranes but there is little AQP-1 immunostaining observed along the basal membrane of the epithelial cells. Similarly, in animals treated with GnRHa, there was strong, apical immunolocalisation of AQP-1 to the efferent duct epithelium and a weaker lateral staining which was at a level comparable to control animals (Figure 6.3 panels c and d). In contrast, treatment with the potent oestrogen DES (10μg/injection) induced a gross reduction in the level of AQP-1 immunostaining (Figure 6.3 panel e).In many ductules, AQP-1 positive cells were only apparent at high power magnification (panel f). There was a dramatic loss of both the apical and lateral membrane immunostaining observed in control animals (panel f). In animals treated with the lower doses of DES (1.0 and 0.1 μg/injection; panels g, h and panels i and j respectively) a sharp reduction in the level of AQP-1 immunostaining was observed when compared with control animals. The reduction observed with the lower doses of DES was not as severe as that observed after treatment with the highest dose (10μg/injection) of DES. At higher magnification, lateral cell membrane staining was still evident but was much more pronounced after treatment with 0.1 μg/injection of DES (panel j) than with 1μg/injection of DES (panel h). Neonatal treatment with ethinyl oestradiol (10μg; Figure 6.4 panels a and b induced a comparable reduction in AQP-1 immunostaining to that observed after treatment with 10μg DES (Figure 6.3 panel e). To illustrate more effectively the reduction in AQP-1 immunostaining after neonatal exposure to potent oestrogens (DES or ethinyl oestradiol at 10μg/injection) a fluorescent image

captured using a confocal microscope by Dr James Evans is shown in Figure 6.5. AQP-1 immunofluoresence appeared green and the nuclei were stained red by the addition of propidium iodide to the mounting medium. Panel (a) illustrates an efferent ductule from a control animal in which AQP-1 immunostaining on both the apical and lateral membranes are clearly observed. In contrast, panel (b) demonstrates the stark loss of AQP-1 immunolocalisation from both the apical and lateral membranes in a DES treated animal. The loss of the columnar epithelial cell appearance is also highlighted by this photomicrograph.

	Day 10		Day 18		Day 25		Day 35		Day 75	
Treatment	AQP-1	n	AQP-1	n	AQP-1	n	AQP-1	n	AQP-1	n
Control	+++	5	+++	11	+++	20	+++	6	+++	6
Control -SF	/	/	+++	13	/	/	/	/	/	/
GnRHa	/	/	+++	12	+++	8	+++	3	+++	5
DES 10µg	+	4	±	14	+	9	++	6	+++	6
DES 1µg	/	/	+	10	+±	4	+++	5	+++	4
DES 0.1µg	/	/	+	10	+±	4	+++	4	+++	4
EE 10µg	/	/	+	4	±	7	/	/	/	/
Genistein 4mg/kg	+++	5	+++	14	+++	7	/	/	+++	5
Bisphenol A 0.5 mg	+++	4	+++	6	+++	5	+++	7	+++	3
Octylphenol 2mg	/	/	++	8	++	6	/	/	/	/
Parabens 2mg/kg	/	/	++	6	/	/	/	/	/	/
Tamoxifen 2mg/kg	/	/	+±	5	/	/	/	/	/	/

Table 6.1 Changes in Aquaporin-1 Immunostaining in Efferent Ducts in Response to Neonatal Oestrogen Treatment

Subjective comparison of changes in AQP-1 immunostaining in the efferent ducts of control and treated animals at 10, 18, 25, 35 and 75 days of age. Key: +++, control level of AQP-1 immunostaining; ++, reduced level of immunostaining; +, major reduction in the level of immunostaining; ±, occasional cells with immunostaining.

Figure 6.4 panels c and d, demonstrate the effect of neonatal treatment with the oestrogen receptor antagonist/agonist tamoxifen on the efferent duct epithelium and on the level of AQP-

1 immunostaining. Tamoxifen also induced a sharp reduction in the level of AQP-1 immunostaining (panel c) to a similar extent to that observed after injection of 1μg DES (Figure 6.3, panel g). The higher magnification shown in panel (d) demonstrates the reduction in epithelial cell height induced by this treatment and also the loss of lateral membrane immunostaining for AQP-1. AQP-1 immunostaining was also performed on 18 day old animals after treatment with the weakly oestrogenic compounds octylphenol (panels e and f), bisphenol A (panel g and h), butyl parabens (panel i and j) or genistein (panel k and l). None of these treatments induced gross reductions in AQP-1 similar to those induced by DES or ethinyl oestradiol but some of the treatments consistently appeared to show slight reductions in the level of immunostaining as judged subjectively.

Thus, when compared to the control slides, both octylphenol (Figure 6.3, panel e) and butylparabens (panel i) appeared to cause a slight reduction in AQP-1 immunostaining and, at higher power, there was very little lateral membrane staining for AQP-1 (panels f and j). Animals treated neonatally with bisphenol A (panels g and h) or genistein (panels k and l) did not show any significant change from control animals. However, at higher power magnification, ducts from animals treated with either bisphenol A or parabens had lateral membrane AQP-1 immunostaining in the efferent duct epithelium (panels h and l respectively).

The AQP-1 immunostaining data for 25 day old animals is tabulated in Table 6.1. Control animals showed a similar level of immunostaining to those assessed at day 18 postnatal. Similarly, there was very little difference in any of the DES or ethinyl oestradiol treatment groups which all appeared very similar to the data shown for day 18. DES still induced a dose-dependent reduction in AQP-1 immunoexpression. Neonatal treatment with genistein, bisphenol A or octylphenol were again identical to the data presented for day 18.

By days 35 and 75 of age, many of the animals showed a less severe response after neonatal oestrogen treatment (see Figure 6.6). Photomicrographs of ducts from 35 day control rats and from animals treated neonatally with GnRHa or 10μg DES are shown in Figure 6.6 (panels a-c respectively). Data from other treatment groups sampled at 35 days of age are reported in Table 6.1. The control and GnRHa treated animals had similar levels of AQP-1 immunostaining (panels a and b respectively). By 35 days of age, animals which were treated neonatally with 10μg DES showed an increase in the level of AQP-1 immunostaining compared to the levels in similarly treated animals at 18 and 25 days of age. This coincided with an increase in epithelial cell height compared with the previous ages though this was still

significantly reduced compared to control levels. Some epithelial cells appeared to show increased basolateral immunostaining for AQP-1. The lower panels (d-i) represent respectively, data from adult animals assessed at 75 days of age comprising control rats, or animals treated with genistein, bisphenol A, DES 10μg/injection, DES 1μg/injection or DES 0.1μg/injection. It was quite clear that the level of AQP-1 immunostaining was very similar between all treatments and that even animals treated with 10μg DES showed detectable AQP-1 immunoexpression on the apical membrane of efferent duct epithelial cells which was not dissimilar to control levels.

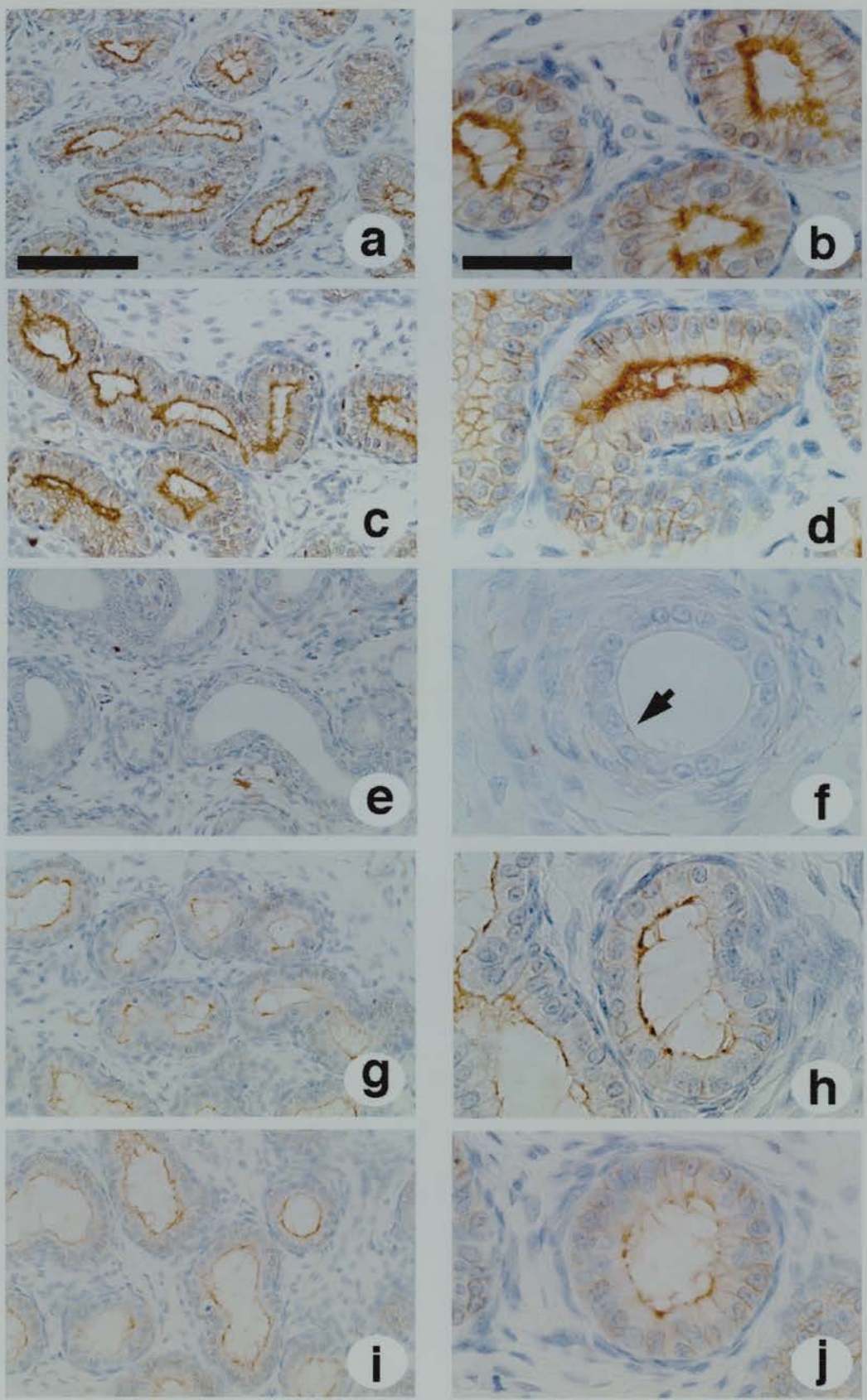

Figure 6.3 Comparison of Aquaporin-1 Immunoexpression in the Efferent Ducts at Postnatal Day 18 After Neonatal Oestrogen Treatment

Immunoexpression of AQP-1 in the efferent ducts at postnatal day 18 in control rats (a, b) and animals treated neonatally with GnRHa (c, d), DES (10, 1.0 and 0.1μg/injection; panels (e, f), (g, h) and (i, j) respectively). Panels (a, c, e, g, f) are shown at x400 and the scale bar in panel (a) denotes 100μm. Panels (b, d, f, h, j) are shown at x1000 and the scale bar in (b) denotes 20μm. The arrow in panel (f) illustrates a cell with AQP-1 immunostaining.

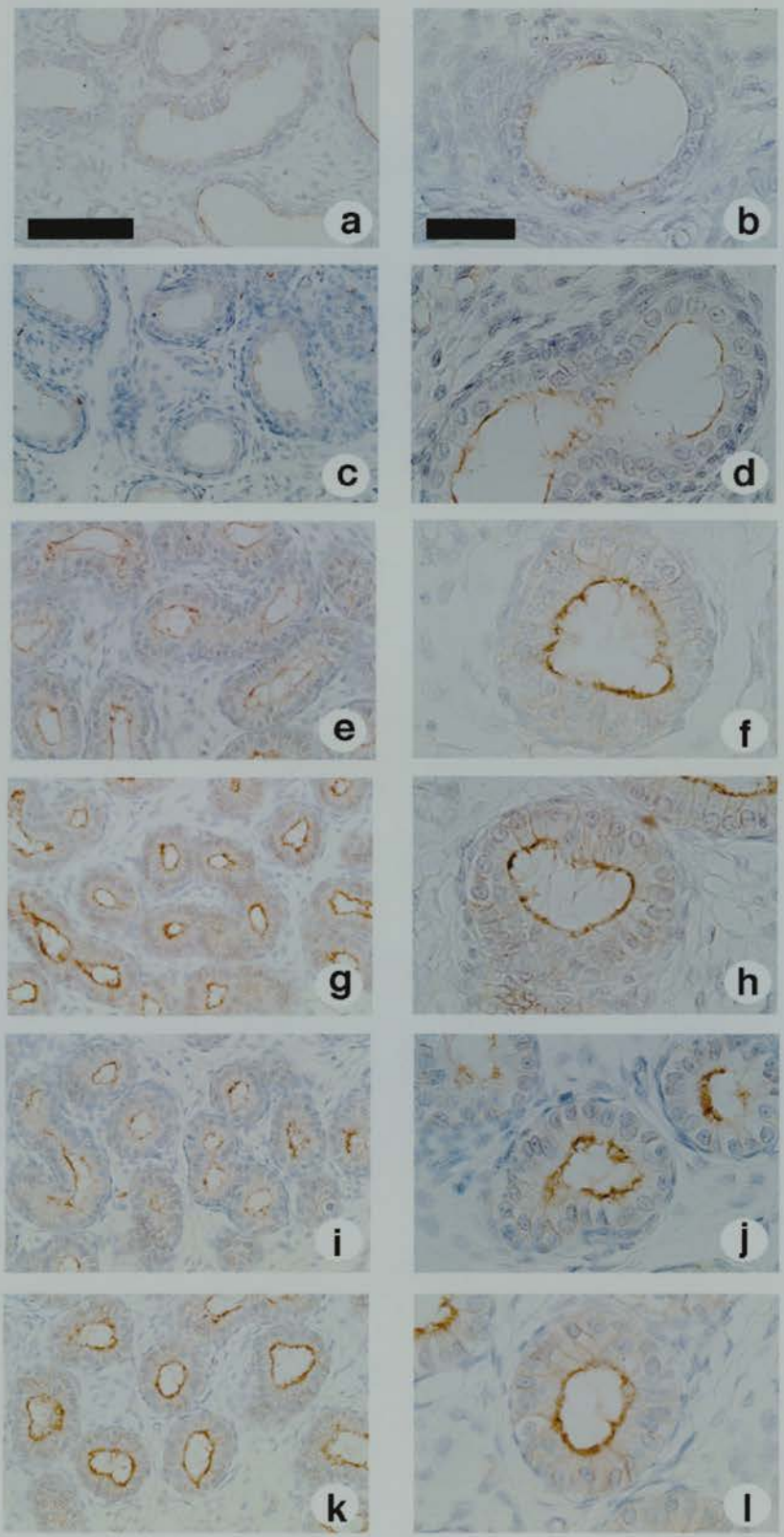

Figure 6.4 Comparison of Aquaporin-1 Immunoexpression in the Efferent Ducts at Postnatal Day 18 After Neonatal Oestrogen Treatment

Immunoexpression of AQP-1 in the efferent ducts at postnatal day 18 in animals treated with ethinyl oestradiol (a, b); tamoxifen (c, d); octylphenol (e, f); bisphenol A (g, h); butyl parabens (i, j) or genistein (k, l). Panels (b, d, f, h, j, l) are shown at x1000 and the scale bar in (b) denotes 20μm.

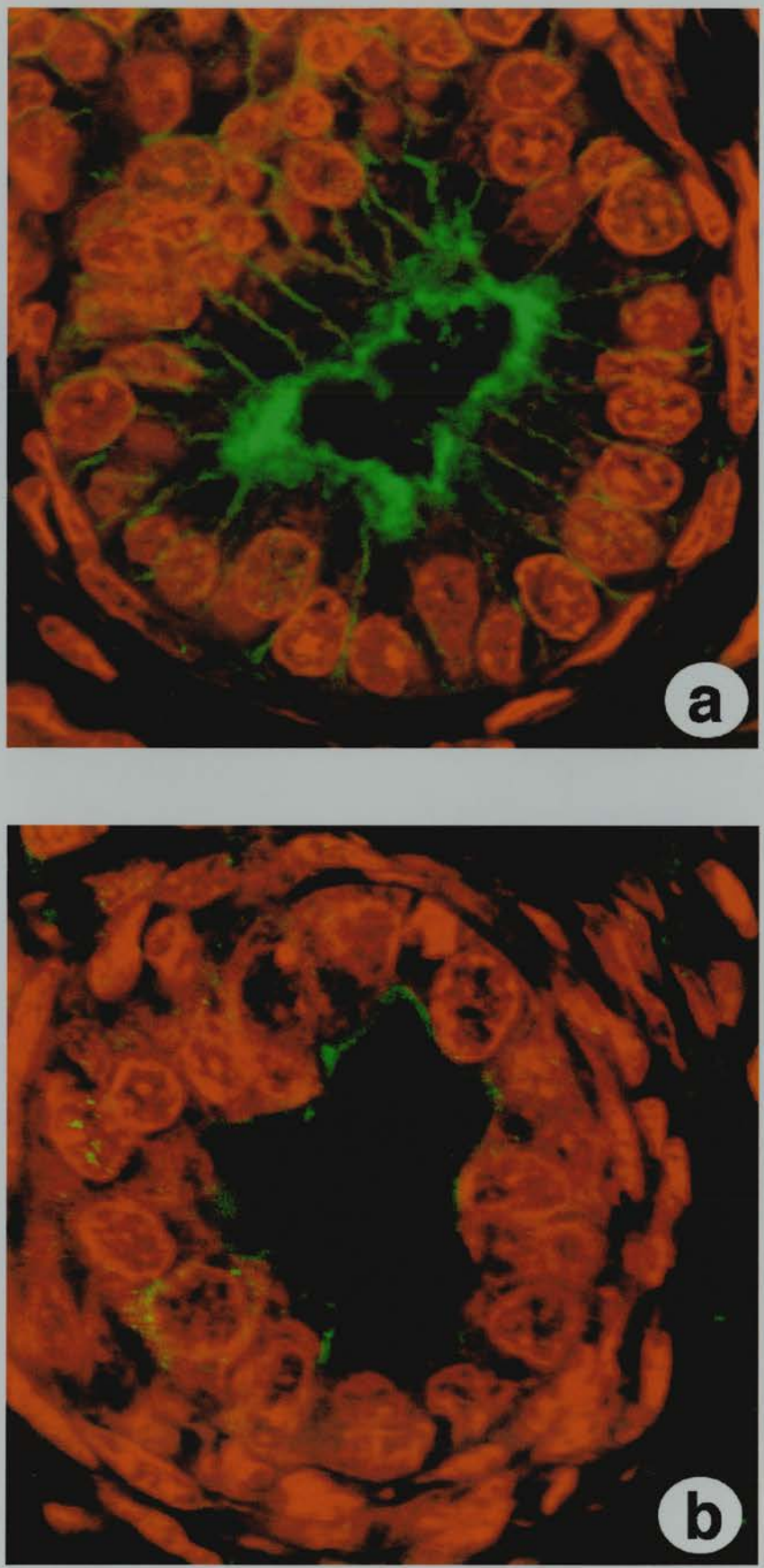

Figure 6.5 Fluorescent Immunostaining of Aquaporin-1 at day 18
Immunoexpression of AQP-1 visualised in the efferent ducts at day 18 in a control rat (a) and a rat treated neonatally with ethinyl oestradiol (10μg/injection) (b). AQP-1 is stained green and the cell nuclei are stained red by propidium iodide.

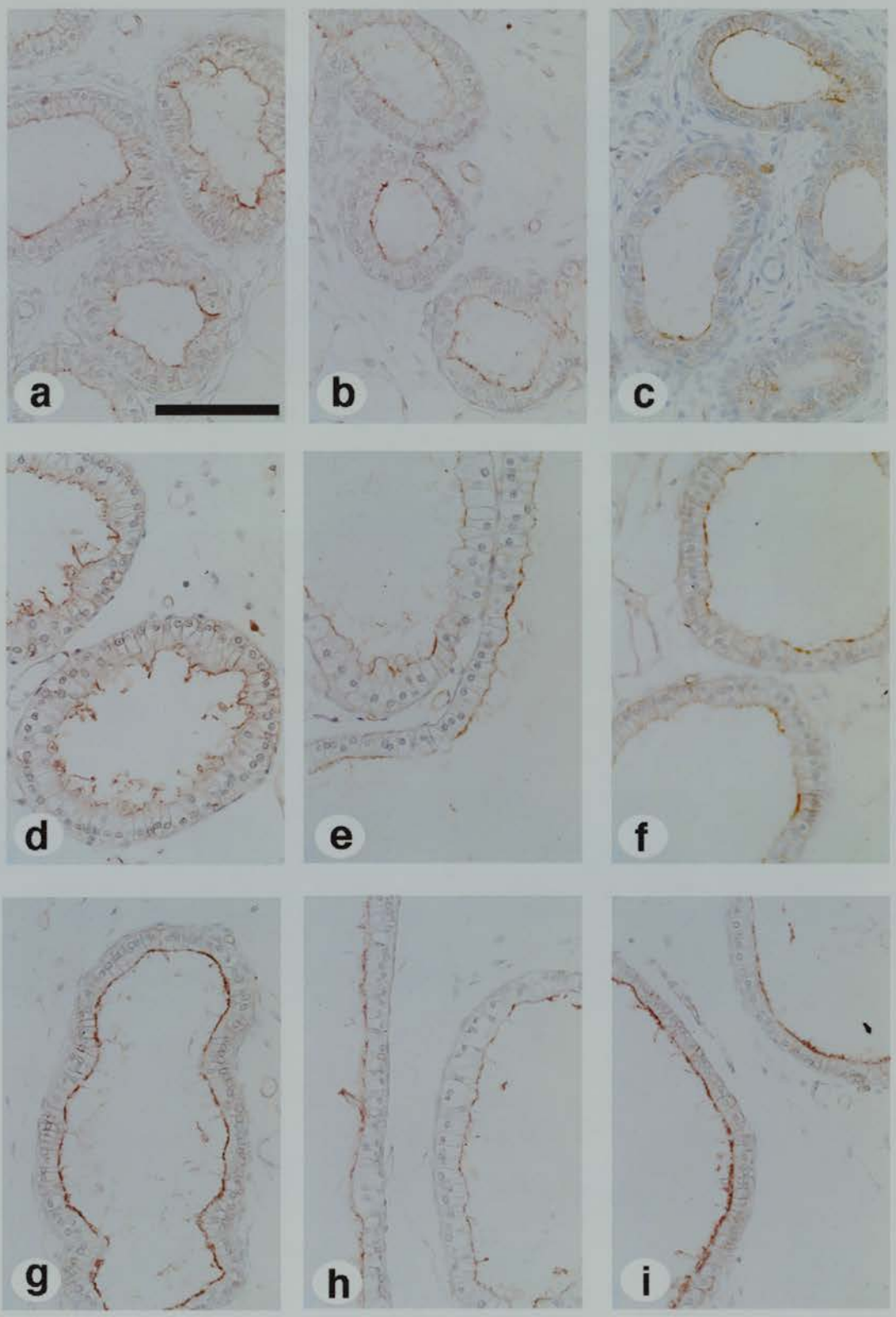

Figure 6.6 Comparison of Aquaporin-1 Immunoexpression in the Efferent Ducts at Postnatal Days 35 and 75 After Neonatal Oestrogen Treatment

Immunoexpression of AQP-1 in the efferent ducts at 35 (a-c) and 75 (d-i) days of age. Results are shown for control animals (a, d) and animals treated neonatally with GnRHa (b), DES (10μg/injection, panels c, g), genistein (e), bisphenol A (f), DES (1.0μg/injection, panel h) or DES (0.1μg/injection, panel i). The scale bar (a) denotes 100μm.

6.3.4 Aquaporin-1 Protein Expression After Neonatal Oestrogen Treatment

The level of AQP-1 protein levels was examined by Western blot. This would establish whether protein was still being synthesised but was not being inserted into the membrane and whether there was a real reduction in AQP-1 protein levels. Figure 6.7 demonstrates that neonatal treatment with DES (10μg) induced a dramatic decline in the level of AQP-1 (lane 8) compared to the level observed in the age matched control (lane 7). This suggests that there is a real decrease in the level of AQP-1 protein after the administration of a high dose of DES. However, it is possible that the AQP-1 protein is synthesised but is folded incorrectly and is no longer recognised by this antibody. This possibility has not been addressed.

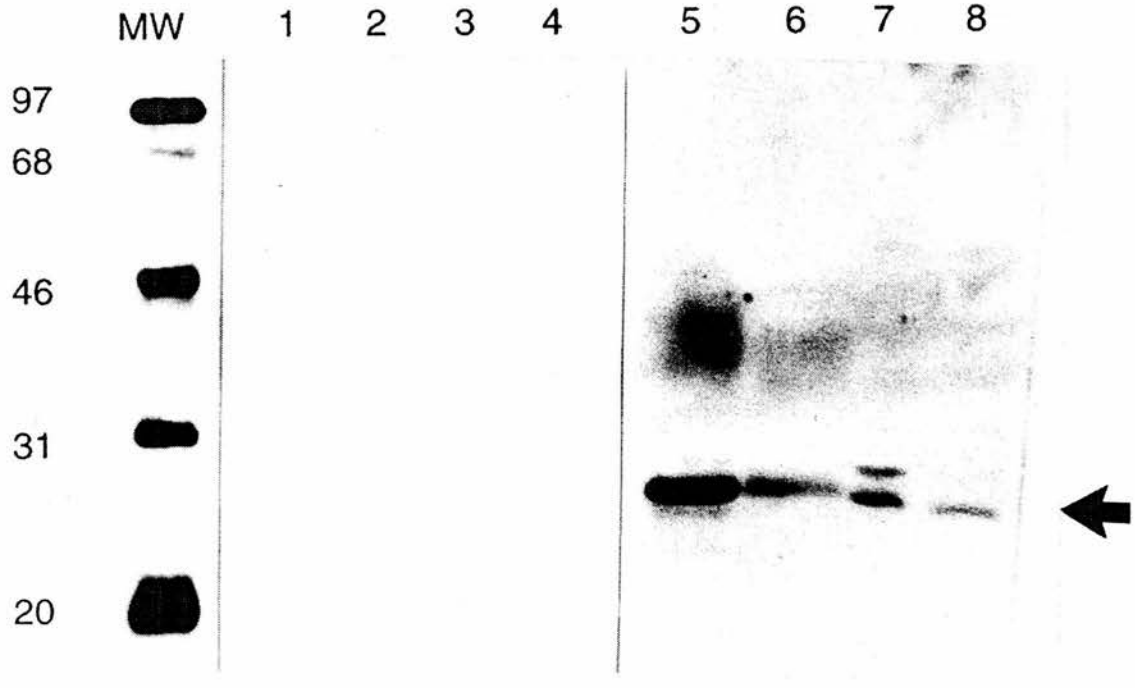

Figure 6.7 Western Blot Analysis of Aquaporin-1 Protein Levels After Neonatal Oestrogen Treatment

This figure demonstrates a specific 28 KDa band localised to both the kidney and efferent ducts. Lanes 1 and 5 contain adult efferent duct protein; lanes 2 and 6 were loaded with adult kidney protein; lanes 3 and 7 contained control efferent duct protein from an 18 day old control rat and lanes 4 and 8 were loaded with protein from a neonatally treated (DES 10μg/injection) rat at day 18 postnatal. Lanes 1-4 were incubated with pre-immune serum whilst lanes 5-8 were incubated with the AQP-1 antiserum.

6.3.5 Aquaporin-1 Immunoexpression in Adult Rat Efferent Ducts After Treatment with GR40370D

Short term treatments were performed on adult rats to determine if the loss of AQP-1 immunoexpression and/or change in epithelial cell height induced by DES treatment might be a consequence of the DES-induced distension of the efferent duct lumen as opposed to a specific effect of oestrogen treatment. The compound GR40370D has been shown to induce distension of the rete testis and efferent ducts and so animals were killed at either 4 or 21 hours post-treatment with this compound (see Section 6.2.1.1). The efferent duct epithelium was examined for both changes in cell height and AQP-1 immunostaining. To ensure that similar regions of the efferent ducts were compared, Figure 6.8 shows photomicrographs that were taken at the rete testis/efferent duct junction.

Figure 6.8 panel (a) illustrates a high power oil immersion photomicrograph of a control section of efferent duct with a connecting section of rete testis epithelium. The characteristic columnar epithelium was evident as was the apical immunolocalisation of AQP-1 to the efferent duct epithelial surface. Panels (b and c) illustrate similar areas 4 and 21 hours after the administration of GR40370D respectively. As there was essentially no difference between these two time points they are described together. It was clear that the administration of GR40370D induced a reduction in epithelial cell height but this was not associated with a loss of apical AQP-1 immunolocalisation although there did appear to be a reduction in the level of lateral membrane localisation. Nor did the distension of the ductule lumen result in complete loss of the apical cytoplasm as is found in DES-treated rats at days 18-25. This treatment suggests that distension *per se* and reduced epithelial cell height do not necessarily induce a loss of AQP-1 immunostaining. However, the rats were not assessed at any later time point to determine if AQP-1 levels remained at control levels or were reduced after a longer period of distension.

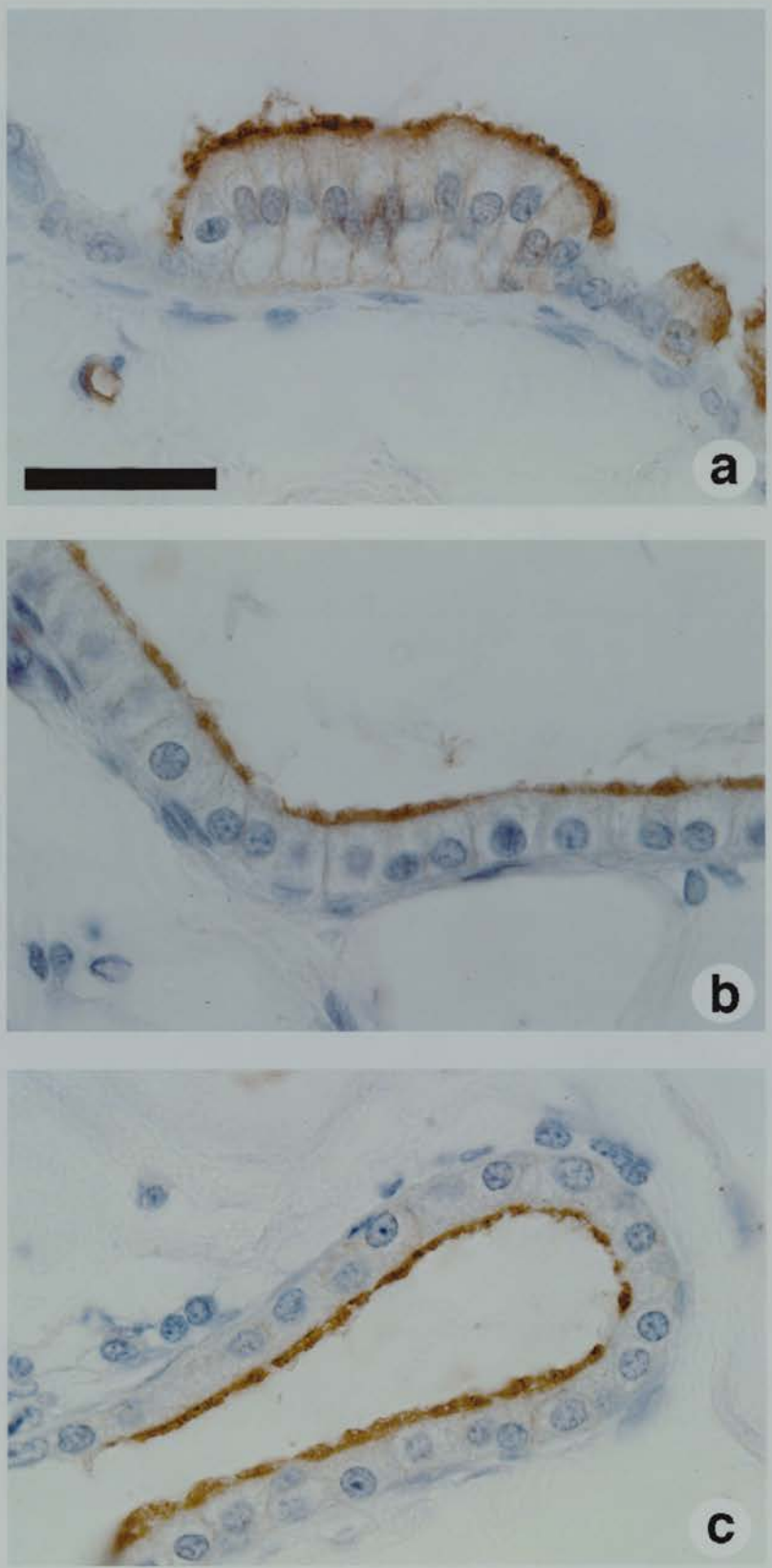

Figure 6.8 Comparison of Aquaporin-1 Immunoexpression in the Efferent ducts of AdultRats After trewatment with GR40370D
Immunoexpresion of AQP-1 in the epithelial cells of the effetent ducts of control rat 9a0 and from animals treated with GR040370 either 4 (b) or 21 (c) hours earlier. The scale bar shows 20µm.

6.3.6 Aquaporin-1 Immunoexpression in Efferent Ducts of Adult Oestrogen Receptor-α Knockout (ERKO) and Wild Type Mice

Tissue sections of testis and efferent ducts from wild type and ERKO mice were kindly gifted by Professor Rex Hess (Dept. Vet. Biosciences, University of Illinois, USA). A similar phenomenon of efferent duct distension has been described in male ERKO mice which are infertile (Hess *et al.*, 1997). It was important to determine whether these mice, which have no functional ERα, also had changes in the level of AQP-1. Figure 6.9 illustrates the immunolocalisation of AQP-1 to a control mouse kidney (panel a) which was performed to ensure that a pattern of immunostaining could be reproduced with the antibody which was comparable to that obtained for rat and marmoset tissue. Panel (a) demonstrates AQP-1 immunolocalisation to the thin descending loop of Henle. Panel (b) demonstrates a similar section of mouse kidney incubated with the pre-immune serum. Panels (c and d) are images of the proximal region of the efferent ducts (i.e. nearest the rete testis) whereas panels (e and f) show the conus region of the efferent duct (i.e. nearest the epididymis). Panels (c and e) show a section from a wild type mouse whereas panels (d and f) are from an ERKO mouse. Comparison of panels (c) and (d) illustrate 3 features of the ERKO phenotype, 1. the efferent ducts were distended 2. there was a loss of AQP-1 immunostaining from the apical membrane, and 3. there was a dramatic reduction in efferent duct epithelial cell height. These three features are identical to the phenotype observed after neonatal DES and ethinyl oestradiol treatment (10μg/injection), as described above. However, when the distal region of the efferent duct was examined in the ERKO mouse (panel f) none of these features were present and the ductules appeared similar to the wild type animal (panel e), except that epithelial cell height still appeared reduced. AQP-1 was only reduced in the proximal region of the efferent ducts which were also the only region which displayed fluid retention. The rete testis of these animals was not present on these slides so it was not possible to determine whether this structure had shown any signs of fluid accumulation but published data has shown gross distension of this region (Lubahn *et al.*, 1996). The fluid distension in the ERKO mouse appeared more localised that after neonatal treatment with potent oestrogens.

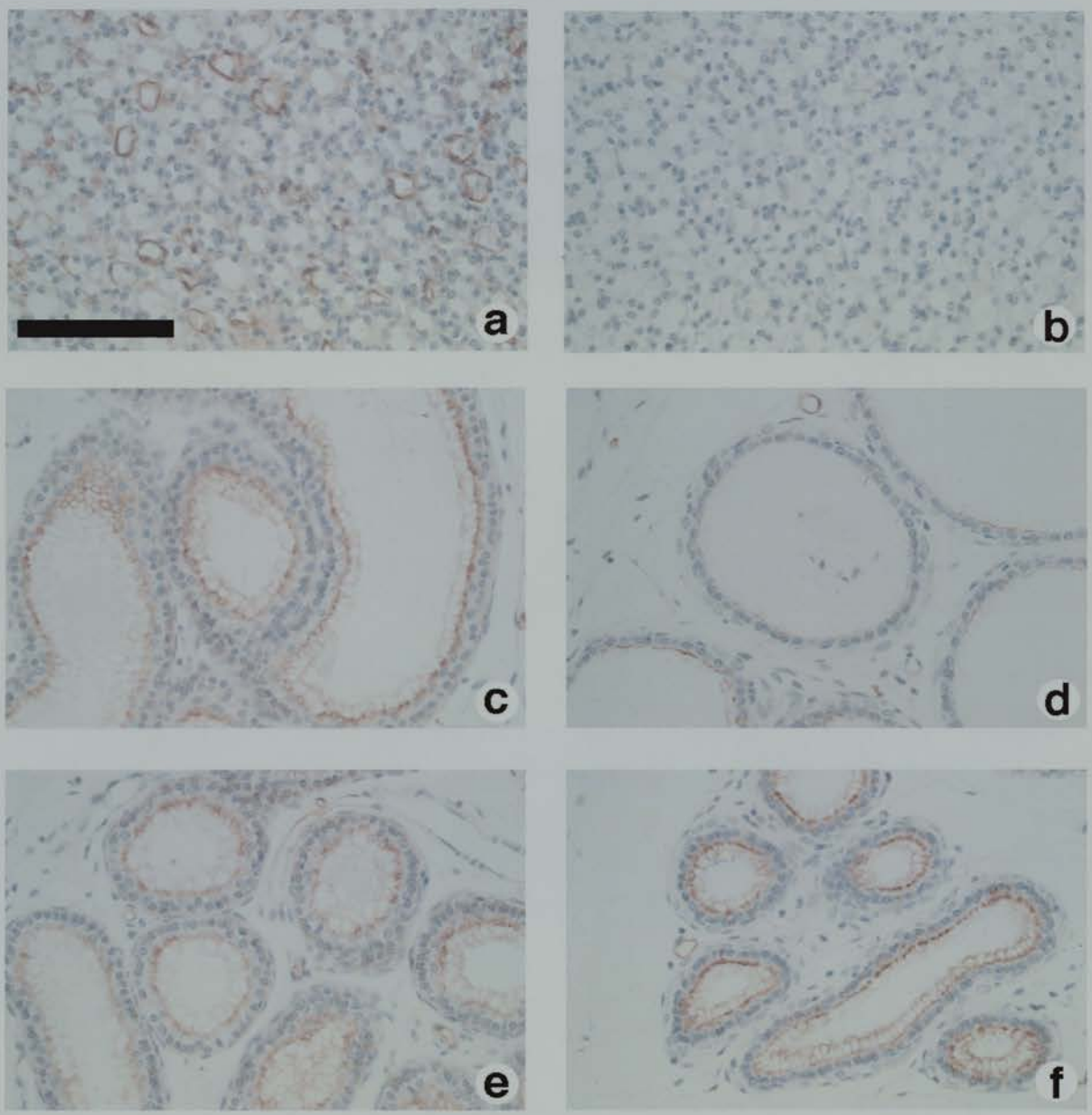

Figure 6.9 Comparison of Aquaporin-1 Immunoexpression in the Efferent Ducts of Adult ERKO and Wild Type Mice

Immunoexpression of AQP-1 in adult mouse kidney (a) and in the efferent ducts of wild type (c, e) and ERKO mice (d, f). a section of mouse kidney incubated with pre-immune serum is shown as a negative control (b). Panels 9c, d) show proximal regions of the efferent ducts, while panels (e, f) show distal regions. The scale bar in panel (a) shows 100μm.

6.3.7 Sex Steroid Receptor Expression After Neonatal Oestrogen Exposure

The loss of AQP-1 immunolocalisation in the proximal region of the efferent ducts of the ERKO mouse, which has no functional ERα, prompted the question of whether there was any relationship between sex steroid receptor expression and the changes in AQP-1 expression. The expression of the AR and oestrogen receptors α and β were assessed in the efferent ducts from 18 day old rats treated neonatally with vehicle (control), GnRHa or DES (10μg/injection). This time point was chosen to assess sex steroid receptor expression as it showed the maximum reduction in both AQP-1 protein levels and epithelial cell height. Figure 6.10 demonstrates the comparison between the expression of these 3 sex steroid receptors in the treatment groups mentioned above. Panels (a-c) illustrate ERα immunostaining, while panels (d-f) demonstrate ERβ immunolocalisation and panels (g-i) demonstrate AR immunocytochemistry. Panels (a, d, g) are tissue sections from a control rat, whilst panels (b, e and h) are efferent ducts from a GnRHa treated animal and panels (c, f and i) are ductules from animals treated neonatally with DES (10μg/injection).

Figure 6.10 demonstrates that none of the treatments induced any change in the immunoexpression of either ERα or ERβ. Both of theses receptors displayed a characteristic checkerboard expression pattern with some cell types showing positive nuclear staining and others being negative. This was identical to the staining pattern observed in the marmoset efferent ducts described in Chapter 2. However, expression of the AR was altered by neonatal treatment (panels g-i). Panel (g) demonstrates the control pattern of AR expression with strong staining in the nuclei of periductular myoid cells and moderate staining of epithelial nuclei which was similar to that observed for both ERα and ERβ. There was also weak AR staining of the stromal cell nuclei. In animals treated neonatally with a GnRHa (panel h) there is a similar pattern of immunostaining to the control panel (g). The same cell types are stained positive for AR but the intensity of the immunostaining appeared reduced. However, after neonatal DES (10μg/injection) treatment there was a dramatic decrease AR staining (panel i). Weak AR immunostaining was still apparent in the stromal cell layer, but nuclei within both the periductular and epithelial cell layer appeared devoid of AR immunostaining.

The level of AR immunostaining was also altered in other regions of the male reproductive tract (Figure 6.11). This figure demonstrates a more detailed account of the changes in AR at day 18 postnatal.

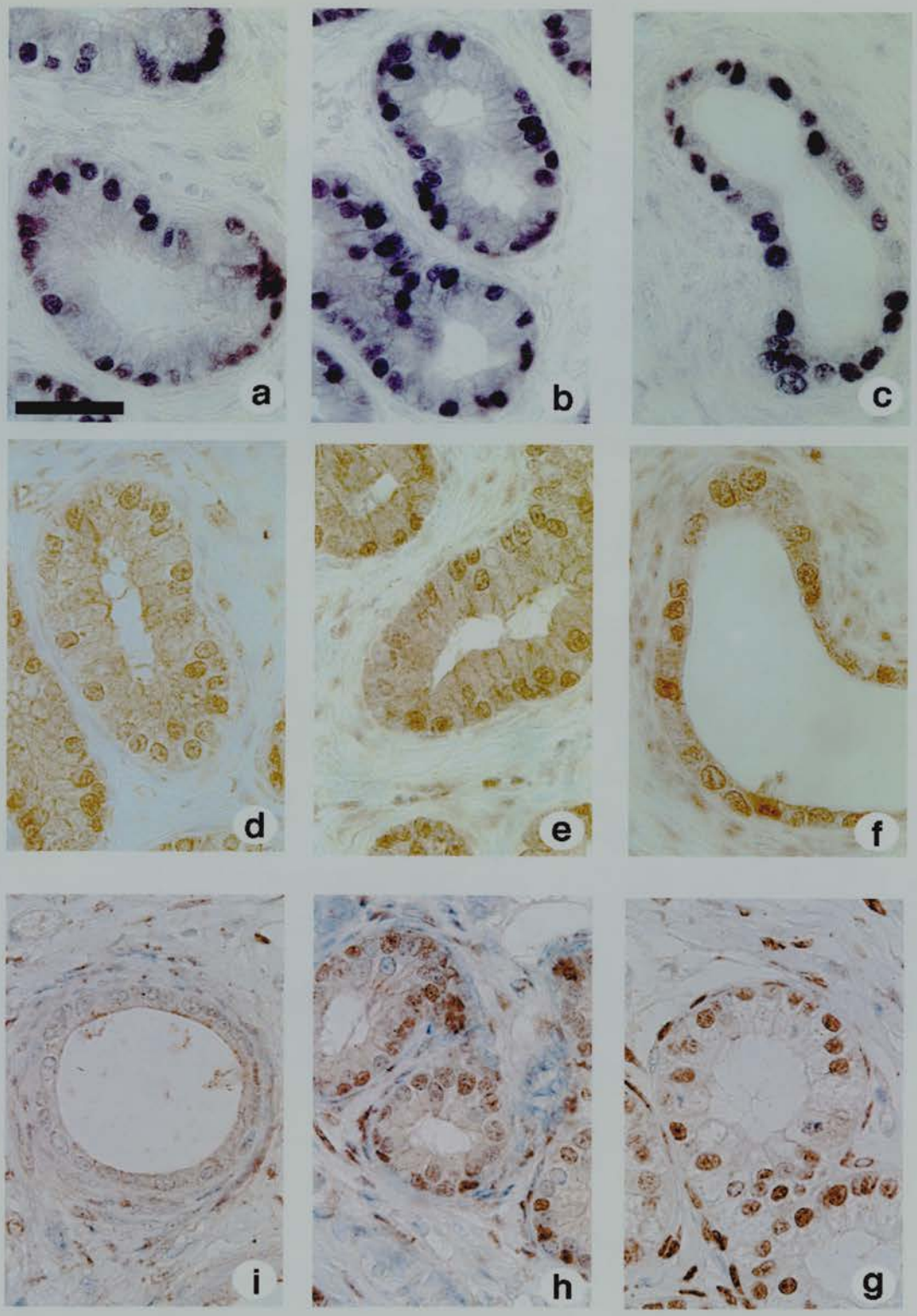

Figure 6.10 Comaprison of Sex Steroid Receptor Immunoexpression in the Efferent Ducts Control and Neonatally treated Rats at Day 18

Photomicrographs of efferent ducts from control rat (a, d, g) and from animals treated neonatally with either GnRHa (b, e, h) or DES (10μg/injection; c, f, i). The tissue sections were immunostined for ERα (a-c) using NBT chromagen; ERβ (d-f) and AR (g-i) were both detected using DAB chromagen. The scale bar shows 20μm.

Panels (a-c), (d-f) and (g-i) are images of testis, efferent duct and caput epididymis from a control, GnRHa and DES (10μg/injection) treated animals respectively. Panel (a) demonstrates the pattern of AR immunostaining within the control testis, which showed strong peritubular myoid cell staining and pronounced Sertoli cell staining which appeared stage specific. AR immunostaining was also localised to Leydig cells of the interstitium. Neonatal GnRHa treatment induced a more variable pattern of AR staining which ranged between control levels to the more extreme phenotype demonstrated in panel (d) after DES treatment. This variability was probably due to either the efficacy of the initial suppression by the antagonist or was due to the faster recovery from suppression in some animals. Both GnRHa and DES caused a reduction in the intensity of the AR immunostaining in all positive cell types but the reduction in intensity was consistently more severe after DES treatment.

AR immunostaining in the efferent ducts was identical to that described for Figure 6.10 (panels g-i) with only DES treatment inducing a complete loss of epithelial cell AR immunostaining. The caput epididymis is the site of the most intense AR immunostaining and this demonstrated in the epithelium of the control epididymis (Figure 6.11 panel c). The epididymal epithelium showed intense AR immunolocalisation in all cell nuclei. A less intense reaction was also evident in the nuclei of the surrounding stromal tissue. After neonatal GnRHa treatment (panel f) the intensity of the AR immunostaining was noticeably reduced in comparison to that observed in control animals. As shown in both panels (c and f), all unstained epithelial nuclei appeared to be those actively undergoing mitosis. There was also a sharp reduction in the intensity of AR immunostaining in stromal cells. Neonatal treatment with DES (10μg/injection) induced a dramatic reduction in the intensity of AR immunostaining in the caput epididymis (in both the epithelial and stromal cell nuclei). Several other morphological changes were also evident (panel i). Firstly, there was gross distension of the epididymal lumen compared to the control tissue (panel c), demonstrating that abnormal amounts of fluid were present within the epididymis. The height of the epithelial cells from the DES treated animal were sharply reduced in height. These changes suggest that unlike the ERKO mouse the abnormalities in fluid dynamics in neonatally DES treated animals are not restricted to the proximal region of the efferent ducts but extend into the epididymis.

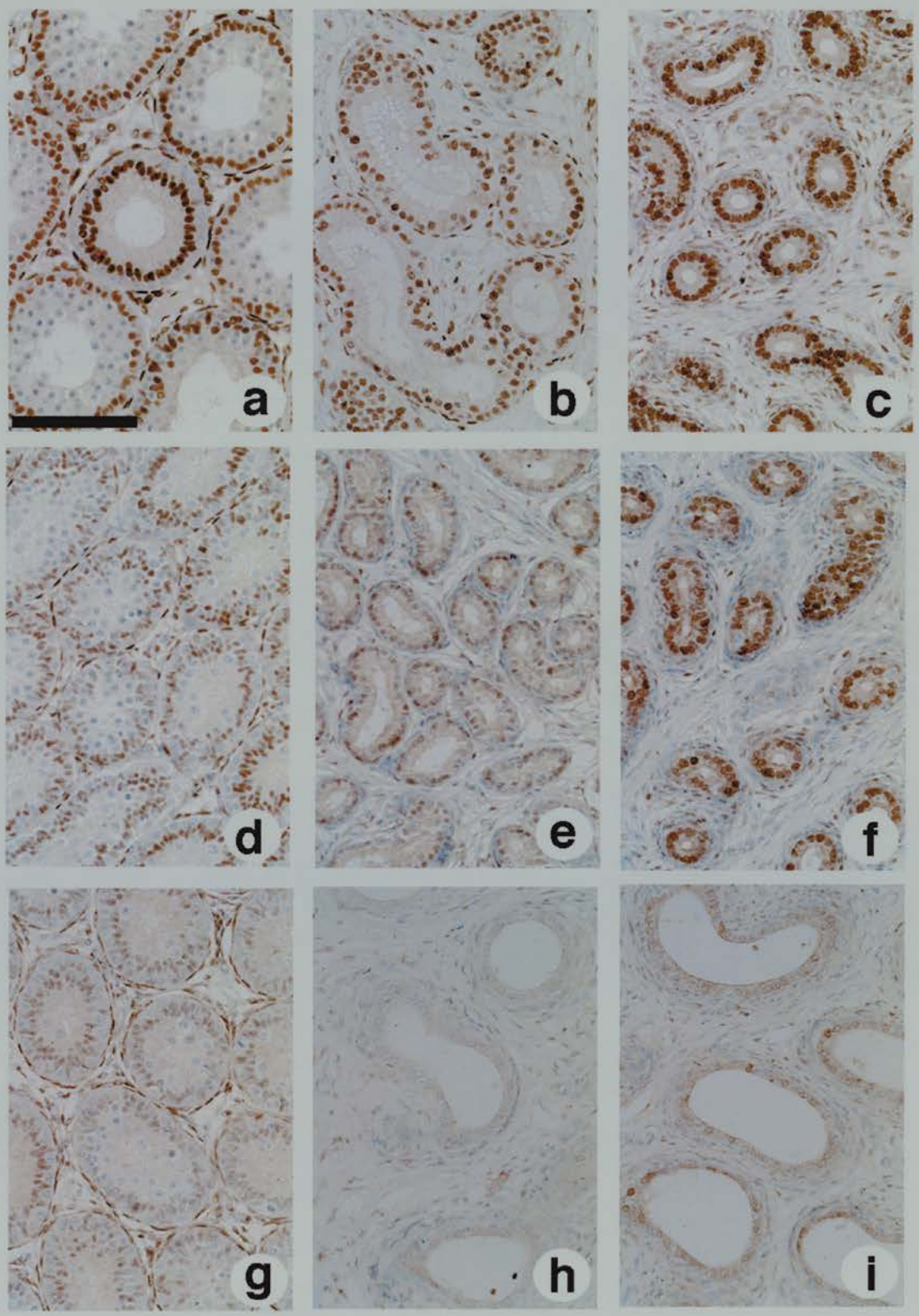

Figure 6.11 Comparison of AR Immunoexpression in the Testis, Efferent Ducts and caput Epdidymis of Control and Neonatally Treated Rats at Day 18 Postnatal
Immunoexpresion of the AR in control rats (a-c), and animals treated neonatally with GnRHa (d-f) or 10μg DES (g-i). The tissues examined were testis (a, d, g); efferent ducts (b, e, h) and caput epididymis (c, f, i). Scale bar in (a) denotes 100μm.

6.3.8 AR Expression in Adult ERKO and Wild Type Mice

The alteration in AR immunoexpression after neonatal exposure to DES (10μg/injection) prompted evaluation of AR immunolocalisation in the reproductive tract of the ERKO mouse to determine whether the lack of functional ERα had any impact on AR immunoexpression within the reproductive tract. In rats, the administration of DES drastically decreased AR immunostaining, possibly mediated by oestrogen receptors, as the data presented in Figure 6.10 demonstrated that DES treatment had no effect on ER immunoexpression.

The data shown in Figure 6.12 demonstrates AR immunostaining within the ERKO mouse. There was no difference in the level of AR immunostaining between the ERKO and wild type mouse although the impression gained was that the immunostaining was more intense in the efferent ducts and caput epididymis of the ERKO mouse when compared to wild type. The loss of AR immunostaining is unlikely to be responsible for the loss of AQP-1 immunostaining observed in either the ERKO mouse or DES treated rat as the level of AR immunostaining is not altered in the proximal region of the efferent ducts in the ERKO mouse. This demonstrates that AQP-1 is not regulated via the AR and that the loss of immunostaining for both AQP-1 and AR after neonatal DES treatment is probably induced by different mechanisms.

6.3.9 Summary of Results

Due to the volume of data contained in this section it seems appropriate to recap the major findings:

1. Neonatal administration of DES/Ethinyl Oestradiol to rats induced:

- luminal distension of the efferent ducts
- reduction in epithelial cell height
- reduction the level of AQP-1 protein levels
- no change in oestrogen receptor (α or β) immunoexpression
- reduction in the immunoexpression of AR

2. ERKO mouse findings:

- reduction in AQP-1 in the proximal efferent ducts which exhibited distension
- no reduction (possible increase) in AR immunoexpression.

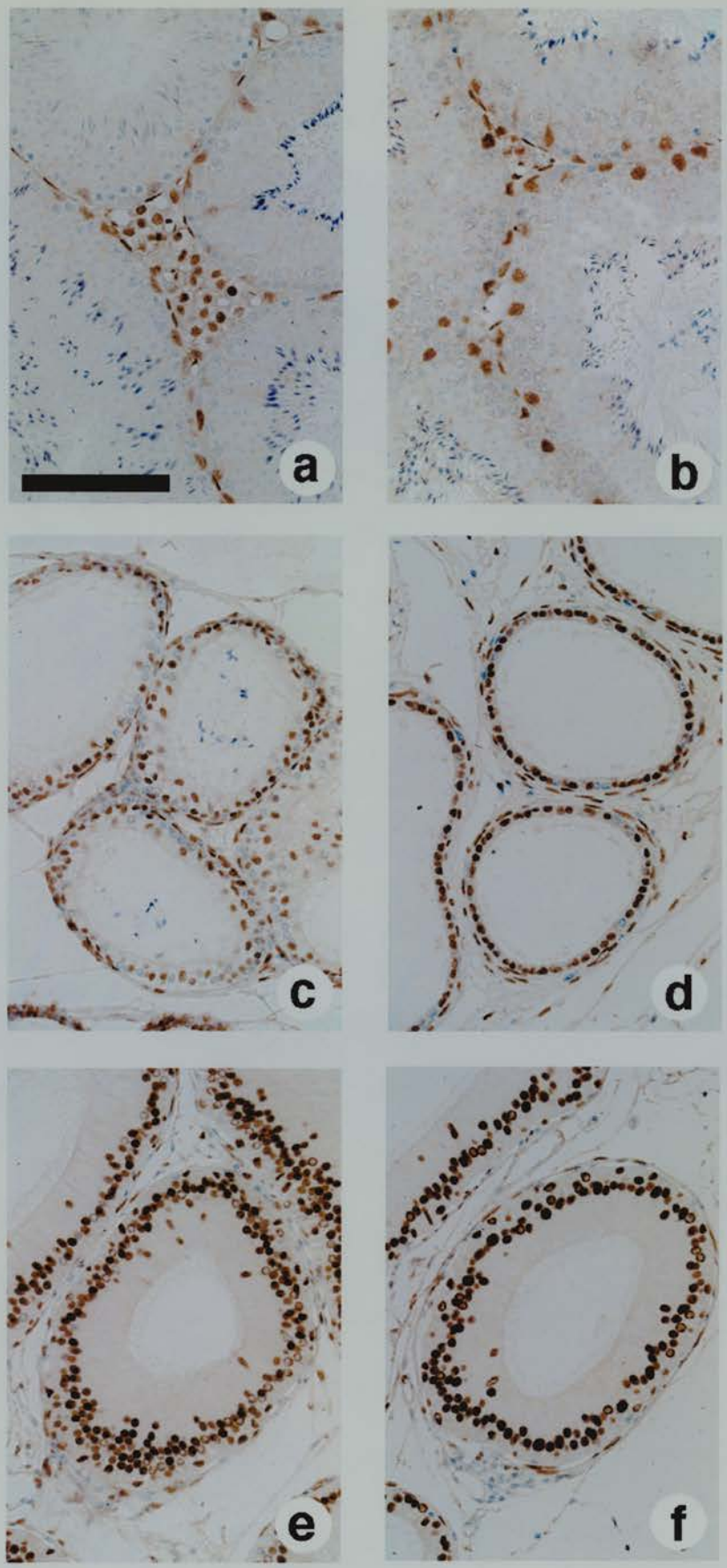

Figure 6.12 AR Immunostaining in Adult Wild Type and ERKO Mice
Immunoexpression of AR in wild type (a, c, e) and ERKO mice (b, d, f). Immunostaining was compared between testis (a, b), proximal efferent ducts (c, d) and caput epididymis (e, f). Scale bar in (a) shows 100μm.

6.4 Discussion

The results presented above demonstrate that neonatal administration of potent oestrogens can induce major effects on the morphology of the excurrent duct system of the male reproductive tract. The severity of these morphological effects was related to the dose of oestrogen administered and appeared to lessen with the time elapsed since cessation of treatment. Functional changes within the excurrent ducts were also identified although only the immunoexpression of AQP-1 was assessed throughout neonatal life and into adulthood so the reversibility of the other changes is not known. The administration of weakly oestrogenic compounds had little effect on either the morphology or functioning (where assessed) of the efferent duct epithelium except for a transient reduction in epithelial cell height observed with some treatments.

6.4.1 Morphological Changes After Neonatal Administration of Potent Oestrogens

There were two major morphological changes to the efferent ducts induced by DES and ethinyl oestradiol (10μg/injection) administration. Firstly, there was an increase in lumen size of the efferent ducts caused by fluid accumulation. In cross sections from control animals the efferent ducts were irregularly shaped but after neonatal oestrogen exposure the cross sections were often spherical and appeared turgid. Secondly, the columnar efferent duct epithelial cells were reduced greatly in height after neonatal oestrogen administration and appeared low cuboidal in shape when assessed at postnatal days 10, 18, 25 and 35. The efferent duct epithelial cells appeared to have lost much of their apical cytoplasm which houses the endocytotic apparatus (Hermo and Morales, 1984). This effect appears to be dose dependent as step-wise reductions in cell height were achieved with increasing dose of DES administered (0.1, 1, 10μg/injection). However, in adulthood, efferent duct epithelial cell height in DES-treated rats was not significantly altered from the control group. This suggests that the morphological alterations observed in the efferent ducts after neonatal oestrogen administration were not permanent.

6.4.1.2 Changes in Efferent Duct Epithelial Cell Height

The reason for the reduction in epithelial cell height after neonatal oestrogen administration is not known but the reduction of epithelial cell height has been studied by researchers who examine epithelial cell function (from various systems) in culture systems. A reduction in cell height *in vivo* is a common problem whenever epithelial cells are removed from their *in vitro* setting and placed in culture. Pertinent data from the literature will be discussed to explore possible mechanisms that may regulate cell height in epithelial cells.

Epithelial cell height is reduced in human efferent duct and epididymal epithelial cells when they are grown in monolayer culture (Raczek *et al.*, 1994). The height of the cultured efferent duct epithelial cells is always lower than the height of the cells from which the cultures were seeded (5.5μm v's ~20μm). However when the cells were grown on filters overlying some non-epithelial portion of the original tissue fragment the cell height was increased to (14μm) (Raczek *et al.*, 1994). This suggests that some of the factors required to maintain the shape of the epithelial cell are released from the cells or matrix surrounding the efferent duct tubules. The degree of epithelial cell height reduction that occurs in this experimental system is not dissimilar to that observed in the *in vivo* studies described in this chapter after neonatal oestrogen treatment (see Section 6.3.2). This might suggest that neonatal oestrogen treatment induces a loss of the factors required to maintain the columnar epithelial cell height which is a characteristic of the differentiated cell.

Raczek *et al.*, also tried to determine if epithelial cell height could be increased by the addition of ^{14}C- testosterone to the cultures (Raczek *et al.*, 1994). They found that in both efferent duct and epididymal epithelial cells, the labelled androgen was principally converted by 17-β oxidation to androstenedione. The epididymal cultures were more effective at converting both of these androgens to 5α-reduced metabolites. However, the addition of ^{14}C- testosterone had no effect on epithelial cell height (Raczek *et al.*, 1994). This study also assessed androgen withdrawal as a mechanism of height reduction in cultured epithelial cells. Cultures were established for 3 days in the presence of androgen but after it was withdrawn there was no observable reduction in cell height. The authors suggested that the short height of the epithelia at the start of the experiment probably prevented any further height reduction following androgen withdrawal (Raczek *et al.*, 1994). However, the changes in epithelial cell height during cell culture may be due to the unphysiological conditions imposed on the epithelial cells during the culture experiments. In these experiments, both the apical and basal surfaces were exposed to the same culture medium, unlike the *in vivo* situation where apical membranes respond to luminal contents.

Epididymal cell height has also been examined in organ culture experiments. Epithelial cells of human epididymal tubules in organ culture, display androgen dependence of cell height (Tezon and Blaquier, 1981) and tissue organisation (Vazquez *et al.*, 1989). When human epididymal tubules were cultured for 8 days, the addition of androgens (testosterone, 5α-dihydrotestosterone or 5α-androstane-3α, 17β-diol) to the culture medium significantly increased the epithelial height. The presence of 0.1μM testosterone in the culture medium during the last 4 days of culture increased epithelial cell height from 9.2μm in control cultures

to 20.2μm after testosterone culture (Tezon and Blaquier, 1981). This suggests that testosterone can regulate epithelial cell height. However, no studies have been performed to directly assess whether oestrogen (which is a metabolite of testosterone) has any impact on epithelial cell height. Its is clear however, from the observations on the ERKO mouse contained in this thesis and the published data that the efferent duct epithelium is reduced in height after the disruption of ERα, yet the ERKO mouse is known to have raised plasma testosterone levels (Eddy *et al.*, 1996), suggesting that oestrogen or ERα-regulated genes may have an influence on epithelial cell height in the efferent ducts.

One other piece of evidence which suggests that epithelial cell height is controlled by either sex steroids or gonadotrophins comes from studies of seasonally breeding animals. In such animals, the reproductive tract shuts down, spermatogenesis ceases and the excurrent ducts regress every year. There is a report on changes in the structure of the efferent ducts of the ground squirrel (*Citellus lateralis*) (Pudney and Fawcett, 1984) which has shown that prior to testicular regression, the cells of the efferent ducts appeared highly differentiated, with distinctive cytological features specialised for resorption and the movement of luminal contents. However, at the onset of testicular regression, the lumen of the efferent ducts became occluded with masses of apical cytoplasm protruding from the epithelial cells and with degenerating cells sloughed into the lumen. At complete regression, the ducts were comprised of cells which were greatly reduced in height and contained fewer organelles. The membrane bound granules characteristic of principal (nonciliated) cells were virtually absent. The exact mechanisms controlling these effects are not understood (Pudney and Fawcett, 1984). However, in our treatments the neonatal administration of a GnRHa did not induce gross changes in cell height or other morphological characteristics of the efferent duct epithelial cells. This suggests that the changes observed in cell height after the administration of oestrogen are either due to the direct action of oestrogen on the epithelial cell or are a secondary consequence of other oestrogen induced changes e.g. reduced androgen action due to a loss of AR expression.

6.4.1.2 Neonatal Oestrogen Treatment and Distension of the Rete Testis and Efferent Ducts

The mechanisms behind the changes in the morphology of the efferent ducts are not clear but there was also evidence of fluid accumulation and distension within the efferent ducts, rete testis and (to a lesser extent) the testis. These changes probably need to be viewed together with the other effects induced by neonatal oestrogen treatment. The results presented in Chapters 5 demonstrated that within the testis there was evidence of germ cell sloughing

around puberty, which became progressively worse and by adulthood. In the adult testis, there were many seminiferous tubules which had few germ cells or which were Sertoli cell-only, especially in the cranial region of the testis underlying the distended rete testis.

The rete testis became grossly distended during neonatal oestrogen exposure and this worsened after the cessation of treatment (until around day 25). At 35 days of age there appeared to be a lessening of this effect but the rete remained distended in adulthood, although at this age the change was not as severe, in relative terms, as that observed at days 18 and 25 postnatal. The most probable cause for the fluid distension is a lack of fluid resorption. However there are no studies which examine whether the efferent duct epithelium performs endocytosis in early neonatal and prepubertal life, and if so what the source of the luminal fluid is. The studies on AQP-1 immunolocalisation (presented in Chapter 4) showed that the efferent ducts/mesonephric tubules always had a patent lumen, and therefore fluid is presumed to be flowing along these ducts in fetal and postnatal life. The most probable source of this fluid is the Sertoli cell but as the Sertoli cell tight junctions have not yet formed there are no lumens within the seminiferous cords giving the appearance that no fluid flow is occurring. This suggests that the composition of the fluid within the postnatal excurrent duct system may be different to the adult, as interstitial fluid can still diffuse into the seminiferous cords altering the composition of the fluid.

The changes in fluid dynamics induced after neonatal oestrogen administration appeared to correlate with changes in epithelial cell height in the efferent ducts. Even during neonatal treatment (postnatal day 10), efferent duct and rete testis epithelial cell heights were noticeably reduced compared to controls. The most severe effects were noted at days 18 and 25 with subsequent recovery to control levels by adult life. The major question is, whether this alteration in epithelial cell height induced a deficiency in fluid resorption or whether fluid accumulation and distension induce alterations in epithelial cell function? Since there are no studies which have directly addressed this question, evidence will be gathered from various *in vitro* organ and cell culture experiments together with studies which have assessed the role of oestrogen within the female reproductive tract to draw comparisons. As most of the published studies have been performed using adult tissue, they may not have direct relevance to our studies, but it is hoped that similar mechanisms occur in the efferent duct epithelial cells during both postnatal and adult life.

In vivo studies have examined the role of sex steroids in controlling fluid reabsorption in the adult efferent ducts (Hansen *et al.*, 1997). Two separate techniques were performed to verify

this data. Initially cannulation studies were performed. Cannulae were inserted in the proximal region of the epididymis and the rete testis to assess the rate at which fluid enters and leaves the efferent ducts. The flow rate in control rats was compared in rats injected for a week with either testosterone propionate (1mg/day), oestradiol benzoate (400μg/day), flutamide (10mg/day) or tamoxifen (1mg/day). Oestrogen administration greatly increased the volume of fluid entering the epididymis from the efferent ducts i.e. fluid resorption was decreased, while tamoxifen and flutamide decreased the amount of fluid entering the epididymis (Hansen *et al.*, 1997). Testosterone caused a small but non-significant increase in fluid resorption. The treatments did not induce any major changes in osmotic pressure in efferent duct fluid suggesting that sex steroid or sex steroid antagonist treatment had little effect on the mechanisms of water transport which arise from solute-solvent coupling (Hansen *et al.*, 1997). The rate of fluid transport was also assessed by microperfusion of individual efferent ducts which produced results consistent with the previous method described above (Hansen *et al.*, 1997). The results suggest that sex steroids are involved in the chronic regulation of fluid resorption by the efferent duct. Exactly how this regulation is achieved is not known but some earlier studies suggest that changes in steroid balance can induce ultrastructural regression within the efferent ducts (see (Hansen *et al.*, 1997)) which could alter membrane folding and other features which influence epithelial transport. The lack of ERα action, as observed in the ERKO mouse has been shown to induce changes in the ultrastructure of the efferent ducts (discussed later) (Nakai *et al.*, 1998).

Whether oestrogen normally acts to induce changes in the ultrastructure of the efferent duct epithelial cells is not clear but the neonatal administration of oestrogen (DES or ethinyl oestradiol) induced changes in efferent duct epithelial cells which suggested almost a complete loss of the apical cytoplasm. There is evidence in the literature that oestrogen can induce rapid, direct effects on the apical membrane of endometrial cells of ovariectomised rats injected intravenously with 17β-oestradiol or DES (Rambo and Szego, 1983; Szego *et al.*, 1988). These effects are rapid and are believed to occur via non-genomic actions of oestrogen at the cell surface. New data from various cell systems suggest that steroids can induce rapid effects through a non-classical, non-genomic action at many sites including the reproductive system (Revelli *et al.*, 1998). Non-genomic effects are suspected when responses to steroid addition are too rapid (seconds to minutes) to be explained via the classical route. Steroid induced effects have been observed in spermatozoa where neither protein or mRNA synthesis occurs and in cell clones devoid of classical steroid receptors. Steroid induced responses have also been elicited when the steroid is coupled to a high molecular weight substance which is unable to cross the plasma membrane. The non-genomic effects of steroid action are not

blocked by classical steroid hormone antagonists (Revelli *et al.*, 1998). Non-genomic effects have been observed after the administration of physiological doses of oestrogen, progesterone and androgen in granulosa and endometrial cells, and in oocytes, spermatozoa and Sertoli cells (reviewed in *Revelli et al.*, 1998).

Non-genomic effects of oestrogen in endometrial cells are thought to be a physiological part of reproductive cycling in mammals, in which the endometrial morphology undergoes cyclical changes in ultrastructure. Endometrial cell morphology has been examined after intravenous injection of 17β-oestradiol or DES (0.5μg/0.25ml per100g body weight) (Rambo and Szego, 1983). In control rats, the endometrial luminal surface was normally characterised by short, sparse microvilli, but 30-60 seconds after oestrogen administration the number of microvesicles on the cell surface was significantly increased and similar results were produced after DES administration (Rambo and Szego, 1983). The number and length of microvilli continued to increase up to 7 minutes after oestradiol injection and then underwent a dramatic regression, and within 15-30 minutes there was a distinct diminution of both microvillar length and numbers (Rambo and Szego, 1983). This surface activity was observed again 1 hour after the initial oestrogen stimulus suggesting that the architectural modifications were biphasic (Rambo and Szego, 1983).

Szego *et al.*, used the same model as described above to assess the effect of oestrogen injection on cytoskeletal microtubules (Szego *et al.*, 1988). This study demonstrated that the endometrial cell microtubular apparatus also undergoes a striking biphasic response in number and length that correlates temporally with reciprocal changes in microvesicle activity (Szego *et al.*, 1988). In control endometrial cells, cytoplasmic microtubules were conspicuous in length and number but, after oestrogen administration, there was a rapid and significant decrease in microtubule length and number so that virtually no microtubules were present 80 seconds after oestradiol injection (Szego *et al.*, 1988). Cytoskeletal microtubule structures began to reappear after 2 minutes and increased progressively to control levels by 30 minutes and as with microvilli formation this phenomenon re-occurred after ~1 hour (Szego *et al.*, 1988). The authors suggest that this extensive reorganisation of the cellular architecture is an early response to oestrogen recognition by the target cell but that the effect of the transitory loss of microtubule integrity on the cell is not understood. The reason for the reoccurrence of these effects after 1 hour was not explained by these studies nor whether they occurred via non-genomic interactions.

The idea that steroid hormones may influence the cytoskeletal ultrastructure of the cell is not new. In 1956, a letter to Nature proposed the idea of a 'cytoskeletal hypothesis' of steroid action (Peters, 1956). The basic tenet of the hypothesis was that steroids did not act to regulate one specific step in a reaction or process but had a much wider impact on the cell. They could act through the rearrangement and reformation of the cytoskeletal elements and so co-ordinate the movement of intracellular substances within and across the cell and its membranes (Peters, 1956). The validity of this hypothesis has neither been proven nor refuted but the evidence presented above suggests that one action of steroid hormones may involve the control of cytoskeletal changes at the apical cell surface.

Whether such non-genomic changes are induced by oestrogenic compounds at the apical surface of the efferent ducts is not known. However, the work presented in this thesis has shown that neonatal oestrogen administration induced dramatic changes in cell shape, a loss or major reduction of the apical brush border and lack of fluid resorption (which can best be explained by disruption to the endocytotic pathway). All of these changes are consistent with changes to cytoskeletal elements. Similar apical membrane changes to those induced after neonatal DES administration have been reported in the efferent ducts of ERKO mice (Nakai *et al.*, 1998). However, no alterations in cell shape or apical membrane morphology were detected after the neonatal administration of GnRHa. This suggests that the changes observed in these parameters after neonatal oestrogen administration are due to a direct action of oestrogen on the epithelial cells and are not dependent on gonadotrophin action.

The major component of the cytoskeleton is the microtubule and they are known to be critically involved in the maintenance of epithelial cell polarity and membrane trafficking (Elkjaer *et al.*, 1995). They are hollow tubes comprised of heterodimers of alternating tubulin molecules (α and β) which are bundled in parallel to form a cylinder. Microtubule polymerisation occurs by the addition of tubulin subunits from a tubulin pool and this process can be disrupted by the addition of the anti-mitotic drug colchicine. A study has been performed on the effects of colchicine induced microtubule disruption on the kidney proximal tubule which is an homologous structure to the efferent ducts (Elkjaer *et al.*, 1995). Five hours after colchicine treatment there were virtually no endocytotic invaginations or large endocytotic vacuoles present, consistent with the idea that disruption of microtubule polymerisation blocks endocytosis. There was also a marked reduction in the number of dense apical tubules (the exocytic vehicle for membrane recycling) but an extensive accumulation of small vesicles, suggesting a disruption in membrane recycling (Elkjaer *et al.*, 1995). Immunofluoresence and immunoelectron microscopy revealed colchicine induced changes in the apical membrane

localisation of AQP-1. After treatment, AQP-1 was redistributed to small cytoplasmic vesicles and the apical membrane staining of the kidney proximal tubule epithelium was grossly reduced (Elkjaer *et al.*, 1995). The level of AQP-1 reduction induced by Elkjaer *et al.*, was not dissimilar to that induced in the efferent ducts after DES or ethinyl oestradiol administration (see Figure 6.5). This suggests that microtubules are involved in maintaining AQP-1 in the apical membrane. If the administration of DES or ethinyl oestradiol affects microtubule formation within the efferent ducts, then the fluid build up would be due to a lack of fluid resorption induced by a breakdown in the endocytotic pathway resulting from a lack of microtubule polymerisation. This suggests that the changes in AQP-1 expression could be a result of disruption to the endocytotic pathway.

Studies performed on the efferent ducts of the ERKO mouse have shown that they have impaired endocytosis (Nakai *et al.*, 1998). The epithelial cells were found to be low columnar/cuboidal with shorter microvilli which appeared disorganised and fewer in number than in wild type mice (Nakai *et al.*, 1998). The cytoplasmic components of the endocytotic apparatus were greatly reduced compared to wild type mice e.g. the number of vacuoles was significantly reduced in ERKO mice. Both groups of mice were capable of endocytosis but the amount of material taken up in ERKO mice was reduced (Nakai *et al.*, 1998). The changes induced in the ERKO mouse are consistent with reduced or inhibited endocytosis.

6.4.2 Functional Changes in the Efferent Ducts After Neonatal Administration of Potent Oestrogens

6.4.2.1 Aquaporin 1

The major functional marker addressed in these studies was the expression of Aquaporin-1. This protein was investigated as it is known to act as a water channel and is normally present on the apical brush border of the nonciliated cells of the efferent ducts from fetal to adult life. This made it a good marker which could be assessed at various time points throughout neonatal life. The neonatal administration of DES induced a dose-dependent reduction in AQP-1 immunostaining with the highest dose (10μg/injection) inducing almost complete abolition of immunostaining while the other two doses induced less severe reductions. The loss of AQP-1 immunostaining was also apparent after neonatal treatment with ethinyl oestradiol (10μg/injection) at a similar level to the corresponding dose of DES. The reduction in AQP-1 protein observed by immunocytochemistry was confirmed by Western blotting. The change in AQP-1 protein levels may be interpreted in at least two ways. Firstly, that oestrogen regulates the expression of AQP-1 or secondly, that the accompanying changes in cell morphology and loss of the apical cytoplasm are responsible for the changes in AQP-1

expression (as addressed in the previous section). No change in AQP-1 immunostaining was observed in animals treated neonatally with a GnRHa suggesting that AQP-1 levels are not dependent on gonadotrophins for expression.

Whether oestrogen regulates AQP-1 has not been fully addressed by this study. It is clear that oestrogen induces a reduction in AQP-1 immunostaining and that the level of reduction is associated with the dose of oestrogen administered. The reduction in AQP-1 immunostaining was not permanent and by adulthood the efferent ducts from all treatment groups displayed control levels of protein but the effect of an oestrogen challenge on the efferent ducts of an adult, neonatally oestrogenised rat was not assessed. If this again reduced the level of AQP-1 this would suggest that oestrogen does have a direct effect on AQP-1 immunoexpression. However, when the levels of AQP-1 protein were assessed at day 18 by Western Blot there was a substantial reduction in protein levels detected after neonatal DES treatment (10μg/injection). There was never a complete loss of AQP-1 immunostaining, suggesting that at most oestrogen could repress but not inhibit AQP-1 expression.

There is evidence from studies on other organ systems that AQP-1 expression can be regulated. AQP-1 was shown to be one of the delayed early response genes expressed by cultured fibroblasts after growth factor stimulation (Lanahan *et al.*, 1992). There have also been reports that AQP-1 can be regulated by steroids. In the fetal lung, AQP-1 expression was initially noted on embryonic day 19 (King *et al.*, 1996). The level of AQP-1 increased 5-fold between E19 and postnatal day 1 and this higher level of AQP-1 expression persisted into adult life. Maternal corticosteroids increased AQP-1 protein and mRNA expression in fetal lung. This rapid increase in AQP-1 expression coincides with major physiological alterations in lung development in preparation for parturition (King *et al.*, 1996). There is also evidence which supports the short term regulation of AQP-1 by a membrane trafficking system in secretin-stimulated cholangiocytes (Marinelli *et al.*, 1997).

To determine whether the AQP-1 gene is capable of being influenced by oestrogen, the promoter region of the gene needs to be examined to determine whether there are any oestrogen response elements (ERE), that would allow oestrogen receptor complexes to bind. Oestrogen can induce target gene transcription in at least two ways. Oestrogen receptors can be transactivated by interaction with an ERE which is composed of two hexonucleotide repeats. The ligand bound dimeric oestrogen receptor binds to this region and activates transcription of the target gene (Paech *et al.*, 1997). ERs can also mediate gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and

Jun for transcriptional activation (Paech *et al.*, 1997). The AQP-1 promoter has been examined to find functional response elements and enhancers (Umenishi and Verkman, 1998). The AQP-1 promoter was found to contain various TATA and CCAAT boxes and various other elements including an AP1 site (Umenishi and Verkman, 1998). This suggests that if oestrogen does regulate AQP-1 expression via an oestrogen receptor mediated pathway then this would have to occur via interaction with the AP1 site.

Further evidence which supports the idea that AQP-1 is regulated via ERs were results from animals treated the neonatally tamoxifen which were assessed at day 18 postnatal. Tamoxifen is an ER antagonist/agonist. In the male reproductive system tamoxifen administration induced a reduction in cell height and AQP-1 immunostaining and caused distension of the efferent ducts (a phenotype similar to the administration of 1μg DES) suggesting that tamoxifen works as an agonist in the male excurrent duct system. According to studies examining the activation of oestrogen responsive genes, tamoxifen activates the transcription of genes that are under the control of an AP1 element (Paech *et al.*, 1997).

6.4.2.2 Aquaporin-1 Immunoexpression in GR40370D Treated Adult Rats and ERKO Mice

The results presented in Chapter 6 provide evidence against cell morphology and distension inducing changes in AQP-1 levels. This study illustrated the continued expression of AQP-1 after the administration of the 5HT-1 receptor antagonist GR40370D, a chemical known to induce rete testis distension (Piner, 1997). GR40370D induced a reduction in efferent duct epithelial cell height and caused efferent duct distension but did not induce a reduction in AQP-1 immunoexpression. However, this compound may have induced these changes using different mechanisms to DES and the 5HT-1 receptor antagonist experiment was performed on adult rats so this may not be the most appropriate model system with which to compare the effects observed on neonatal animals.

AQP-1 levels are altered by oestrogen administration, so to determine whether ERα modulates AQP-1 levels, its expression was examined in the ERKO mouse. The ERKO mouse had distended efferent ducts but this was less severe that that induced after DES (10μg/injection). Only the proximal region of efferent ductules nearest the rete testis were distended and the distal conus region of the efferent ducts appeared to have a similar phenotype to wild type mice. AQP-1 immunostaining was reduced only in the proximal region of the ducts which were also distended, but the level of AQP-1 staining in the conus region was similar to wild type mice. This suggests that ERα is only important in controlling fluid dynamics in the

proximal region of the efferent ducts or that the effects on epithelial cells induced by a lack of ERα has induced a reduction in the level of AQP-1 observed at the apical membrane. The phenotype induced after the loss of ERα in ERKO mice and after neonatal DES administration share some similarities therefore the studies examining the male reproductive tract of ERKO mice will be described.

Male ERKO mice are infertile (Korach, 1994) suggesting an essential role for ERα in regulating male fertility. Similar to neonatal DES administration, adult ERKO mice have a reduced testis weight (which is 50% of wild type testis weight) and elevated serum testosterone levels (Eddy *et al.*, 1996). By 20-24 weeks of age the ERKO mouse testes contained many atrophic and degenerating seminiferous tubules. Some seminiferous tubules had dilated lumens, a Sertoli cell-only epithelium or a disorganised seminiferous epithelium with few germ cells (Eddy *et al.*, 1996). The rete testis and efferent ducts were dilated (Eddy *et al.*, 1996; Lubahn *et al.*, 1996) and efferent duct endocytosis was compromised (Nakai *et al.*, 1998). Aside from the increased serum testosterone levels observed in ERKO mice, there appear to be some similarities between the phenotypes induced in the ERKO mouse and after neonatal oestrogen administration. However, there are also notable differences. For example, the distension induced in the rete testis and efferent ducts in DES-treated rats is first evident neonatally rather than postpubertally as in ERKO males and, unlike the latter, does not become progressively worse. In fact, many of the effects induced by neonatal oestrogen administration are reversed by adulthood. Again, DES treatment induced an overgrowth of the rete testis before puberty, whereas no such change has been reported for ERKO males. It is probable that the similarities and differences in the phenotypes induced in DES treated animals and in ERKO mice are probably due to the unphysiological levels of oestrogen in the neonatal period. This may induce changes in fluid dynamics which perhaps would normally only occur after puberty as in the ERKO mouse. Whether the phenotypes displayed by these two models are induced by the same mechanisms is not known.

6.4.2.2 Immunoexpression of Steroid Hormone Receptors in the Efferent Ducts after Neonatal Oestrogen Exposure

The comparison of steroid receptor expression in the neonatally oestrogenised rat was performed at day 18 as this was the age when most severe effects were observed and so any difference in steroid receptor expression would be more likely to be noticed at this age. There was no appreciable change in the immunoexpression of either ERα or ERβ in the efferent ducts after neonatal oestrogen treatment. However, when immunostaining for the AR was performed a major reduction in the level of immunostaining was observed. On further

examination it was noted that AR immunostaining was decreased throughout the testis, rete testis, efferent ducts and most noticeably in the caput epididymis which normally has the most intense immunoreaction of AR at this age. This result suggests that oestrogen can regulate AR expression. There was no reduction in AR immunostaining within the testis, efferent ducts or epididymis of the ERKO mouse suggesting that oestrogenic compounds such as DES or ethinyl oestradiol can act directly to suppress AR expression and that an interaction with ERα is not required.

A recent report by Yeh et al., has shown that 17β-oestradiol (E_2) can activate AR target genes through a direct action of E_2 on the androgen receptor. Steroid induced gene transcription occurs when the activated receptor (i.e. ligand bound) interacts with a hormone responsive element and this complex communicates with the cellular transcriptional apparatus to induce or repress the expression of target genes. Coactivators and repressors are involved in the transactivation and may function as a bridge between the receptor and the basal transcriptional factor complex. A coactivator of the AR (ARA_{70}) has been isolated and shown to activate AR target genes in the presence of 10nM E_2 but not in the presence of DES (Yeh *et al.*, 1998). The authors suggest that E_2 may represent a natural ligand for AR which may have an important role in androgen action (Yeh *et al.*, 1998). Other coactivators and repressors also exist which may allow other oestrogenic compounds to induce the transcription or repression of other AR target genes. One other report exists in the literature that suggests that oestrogen can directly effect AR transcription (Cardone *et al.*, 1998). In primary cultures of lizard testis, oestradiol has been shown to autoregulate ER mRNA and downregulate AR mRNA (Cardone *et al.*, 1998). The downregulation of AR mRNA was reversed by the addition of the antioestrogen ICI 164 384 and cycloheximide (to inhibit protein synthesis) (Cardone *et al.*, 1998). These two studies suggest that there is an intimate link between the actions of oestrogens and androgens which may prove important in explaining the changes in AR immunostaining observed in the studies presented in this thesis after neonatal oestrogen exposure.

6.4.2.3 The Effect of Weakly Oestrogenic Chemicals on the Rat Efferent Ducts

The results described in Chapters 5 and 6 suggest that weakly oestrogenic compounds (octylphenol, bisphenol A, parabens or genistein) as assessed in this study had no major or permanent effects on the male reproductive tract. In the absence of specific biochemical and molecular markers of oestrogen function in the excurrent ducts, the morphological parameters that were found to change after neonatal exposure to potent oestrogens (DES and ethinyl oestradiol) were examined. In contrast to neonatal administration of potent oestrogens, testis

weight was not reduced by the administration of weakly oestrogenic compounds. These chemicals either caused no significant change or induced significant increases in testis weights. The only objective assessment which showed any significant reduction after the neonatal administration of weakly oestrogenic compounds was the measurement of efferent duct epithelial cell height. Transient reductions were observed after treatment with octylphenol and bisphenol A (days 18 and 25 postnatal) and genistein (day 18 postnatal). When epithelial cell height was assessed after day 25 postnatal, none of the weakly oestrogenic compounds had any effect. None of the weakly oestrogenic compounds induced rete testis or efferent duct distension. AQP-1 levels did appear reduced after neonatal treatment with octylphenol when assessed at day 18 and 25 postnatal, although this was a subjective observation and any reduction was marginal when compared to the reductions induced in AQP-1 immunostaining by DES and ethinyl oestradiol.

Since the oestrogenic properties of these chemicals were discovered, researchers have performed further studies to define the biological effects of these chemicals. Octylphenol has been shown to bind to oestrogen receptors and exert oestrogen effects on mammalian cells (Blake and Ashiru, 1997). In the studies described in Chapter 6, it was found to induce transient reductions in AQP-1 immunostaining and efferent duct epithelial cell height. However, octylphenol induced no major effects on fluid resorption suggesting it induced a weak oestrogenic response in the male rat reproductive tract when administered neonatally (at 2mg/day on days 2-12 inclusive). The effect of octylphenol administration to adult male rats has been investigated (Boockfor and Blake, 1997) and was found to disrupt reproductive hormone secretion after the injection of 80mg at 3 times weekly for 1 or 2 months. This treatment was found to induce a decrease in the weights of the testis, epididymides and accessory sex glands (Boockfor and Blake, 1997). In a similar study, the same dose of octylphenol decreased serum levels of LH, FSH and testosterone but induced an increase in prolactin concentrations (Blake and Bookfor, 1997). The effects recorded by these studies, were induced after the administration of huge doses which may demonstrate the oestrogenicity of this compound, but they are not physiologically relevant concentrations. Such studies add little to the debate as to whether environmental levels of octylphenol or other 'environmental oestrogens' are detrimental to reproductive health.

The identification of some parabens preservatives as weakly oestrogenic is a relatively recent finding but no adverse effects on the male reproductive tract were observed in our studies. Butyl parabens is weakly oestrogenic and binds the oestrogen receptor with a binding affinity 1-2 magnitudes lower than nonylphenol and is ~100,000 times less potent than 17β-oestradiol

(Routledge *et al.*, 1998). In our studies, parabens was administered at a dose 18 times lower than octylphenol, so perhaps the lack of observable effects was not surprising.

The other source of environmental oestrogens that has been proposed as possible causal agents in the changes observed in male reproductive health, are those ingested via the diet through the ingestion of plant and microbial oestrogens. The isoflavone phytoestrogen genistein, may be responsible for some oestrogenic effects of soy *in vivo* (Strauss *et al.*, 1998). The administration of the genistein (2.5mg s.c./kg bodyweight) to adult male mice for 9 days induced reductions in both testicular and serum testosterone (Strauss *et al.*, 1998). When genistein was administered to neonatal male mice only high doses (1mg/pup/day) induced prostatic changes (squamous epithelial metaplasia) similar to those induced after DES administration (Strauss *et al.*, 1998).

The evidence regarding biological effects of environmental oestrogens within the published literature is not very strong at present. There are only a handful of studies, which are generally not directly comparable, due to the administration of different doses, routes and ages of administration. The experiments performed in this thesis give little support to the hypothesis that any adverse changes in male reproductive health are due primarily to increased exposure to oestrogenic compounds (either 'environmental' or dietary oestrogens). The marginal and transient effects induced in our studies suggest that chronic exposure to very high levels of weakly oestrogenic compounds would be required to induce any adverse effect on the male reproductive system. However, until we are sure exactly what exposure levels in the environment are, and how these chemicals can or do interact with one another, we should not be complacent and should continue to find some quantifiable oestrogen regulated endpoints that can be used to assess the relative risk of these chemicals to human health.

6.4.3 Summary

The results in Chapters 5 and 6 of this thesis show that neonatal exposure to high levels of oestrogen caused deleterious changes in testis weight and other endpoints. Although these effects persisted after the cessation of treatment, most of the parameters measured except for testis weight and rete testis morphology, had normalised by 75 days of age. Where changes persisted, this was only in animals treated neonatally with high doses of potent oestrogens (DES and ethinyl oestradiol 10μg/injection). The major problem with the parameters assessed in this study is that they were largely subjective and not quantifiable. For example, it is difficult to compare rete testis morphology between animals as cross sections from identical regions of the tissue must be assessed, which is labourious and only gross changes can be

detected with certainty. Similarly, it is also difficult to detect subtle changes in the level of AQP-1 immunostaining, particularly that which might have arisen after neonatal treatment with weakly oestrogenic compounds. The most useful parameters measured were efferent duct epithelial cell height and testis weight. Changes in testis weight are very useful to gauge the overall effects of a treatment. However, administration of other compounds such as GnRHa, produced similar reductions in testis weight to DES (10μg/injection) demonstrating that this parameter cannot be used as a specific endpoint to assess inappropriate oestrogen exposure. The postnatal period is critical for normal testis growth and alterations in Sertoli cell number induced during this time are permanent, whereas other regions of the male reproductive tract appear to be more plastic and show signs of recovery from oestrogenic effects. For example, at 75 days of age, rats treated neonatally with DES (all doses) have shown significant reductions in testis weight, whereas efferent duct epithelial cell height was not significantly different from control values. However, efferent duct epithelial cell height was a useful and objective endpoint for assessing both gross and subtle effects of oestrogens earlier in life and could also be used to monitor the duration of these effects. Although the parameters assessed in these studies, when taken together, give a strong indication of effects induced by oestrogenic chemicals on the reproductive tract, the specificity of these effects remain to be established: can other compounds that are not oestrogenic induce similar changes?

Chapter 7 Epididymal Ion Channel Expression after Neonatal Oestrogen Treatment

7.1 Introduction

In earlier chapters, the importance of proper fluid dynamics was described, showing evidence for the distension and abnormal morphology of the testis, rete testis and efferent ducts after treatment with either DES or ethinyl oestradiol. These changes occurred coincidentally with the disappearance of the water channel protein AQP-1 from the brush border of the efferent ducts. This water channel is not expressed in the remainder of the rat epididymis but has recently been localised to the vas deferens (Andonian and Hermo, 1999). Many ion channels are known to be expressed in the epididymis and fluids are also known to be transported secondary to ion transport. This chapter examines the effect of neonatal exposure to DES, ethinyl oestradiol and GnRHa on the function of selected ion channels in the epididymis. The adult epididymis is an important organ in inducing the maturation of spermatozoa and the maintenance and storage of the mature sperm in a quiescent state. The induction of this immotile state is attained via the steady lowering of the epididymal luminal pH during passage through the epididymis (Hinton and Palladino, 1995). The alteration in pH is referred to as luminal acidification. This fluid becomes neutralised before ejaculation due to the basic secretions from the prostate (Carr and Ascott, 1989). The exact mechanisms responsible for luminal acidification are not fully understood but studies which have used micropuncture and other techniques to examine the ionic composition of epididymal fluid have shown that ion channels must be present on the epididymal epithelial cell membranes to account for the changes in ion flux within the epididymal fluid. Recent studies using immunocytochemistry have identified some of these proteins. Two apical proteins, the Na/H exchanger and H^{+}ATPase have both been implicated in the process of luminal acidification. Aside from luminal acidification, ion channels are also involved in fluid resorption and the maintenance of osmotic balance within the epithelial cell and the luminal environment.

The epididymis is generally regarded as an androgen target organ but there are a few studies which suggest that some of the ion channel functions may in fact be under the control of oestrogens. This chapter initially aimed to localise the chloride channel involved in the pathology of cystic fibrosis (Cystic Fibrosis Transmembrane Conductance Regulator, CFTR). Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder of Caucasians (Rochwerger *et al.*, 1994). CF patients are affected by chronic obstructive lung disease, pancreatic insufficiency and intestinal malabsorption and obstruction as well as reduced or absent fertility in both sexes (Rochwerger *et al.*, 1994).

CFTR is a cAMP-regulated plasma membrane chloride channel which is a member of the ABC (ATP-binding cassette) transporter superfamily. The CFTR protein contains two sets of membrane spanning segments, two nucleotide -binding domains (NBD) and a large intracellular regulatory domain (R domain) which contains the protein kinase A and C sites of the molecule (Tummler and Puchelle, 1997). Opening the CFTR channel requires phosphorylation of the R domain by cAMP- protein kinase A, and ATP binding and hydrolysis by the NBD (Winter and Welsh, 1997). Parts of the transmembrane domains form the channel pore, and the R domain functions as a channel inhibitor until it is phosphorylated and undergoes a conformational change allowing chloride ions to travel through the pore (Tummler and Puchelle, 1997). CFTR expression and functions are regulated by multiple transcriptional and translational mechanisms. Several alternatively spliced isoforms of CFTR have been identified of which at least some are functional and organ specific (Tummler and Puchelle, 1997).

In human female, third trimester fetuses, CFTR expression is present in the cervix and fallopian tubes (Tizzano *et al.*, 1994). This expression pattern gradually decreases during the first year of life. During puberty, CFTR expression reappears strongly in the cervix but at a weaker level in the endometrial epithelium and glands and in the fallopian tubes. In female reproductive tissues, CFTR is thought to play a role in fluid exchange and electrolyte composition, both of which are influenced by the hormonal changes during the oestrus cycle (Tizzano *et al.*, 1994). Female patients with CF have a thick cervical mucus and do not manifest a midcycle surge in cervical mucus electrolyte concentrations as observed in healthy women (Kopito *et al.*, 1973). In healthy cycling rats, CFTR is maximally expressed in the luminal epithelium during pro-oestrus which coincides with a peak in uterine fluid volume (Trezise *et al.*, 1993). Experiments have shown that CFTR is regulated by steroid hormones in rat uterine and oviductal epithelia *in vivo* (Rochwerger and Buchwald, 1993) while no steroid hormone regulation appears to be present in other tissues such as the lung and intestine (Tizzano *et al.*, 1992). The administration of oestrogen to immature and ovariectomised mature female rats was shown to induce CFTR expression (Rochwerger and Buchwald, 1993). Studies have also shown that oestrogen regulates the expression of CFTR *in vitro* using a novel uterine epithelial cell line (Rochwerger *et al.*, 1994). In this cell line CFTR expression was dependent on the presence of oestrogen (at physiological concentrations, 10^{-9} and 10^{-10} M) in the culture medium and no expression of CFTR was observed in cell cultures grown in serum devoid of steroid hormones (Rochwerger *et al.*, 1994).

Most male patients with cystic fibrosis also have impaired fertility due to atrophy or obstruction of the epididymis, vas deferens and seminal vesicles and the resultant azoospermia (Tizzano *et al.*, 1994). The relationship between the CFTR dysfunction and the resulting pathological changes in the reproductive tract are not understood, to the extent that researchers do not know whether these changes are a result of a morphogenetic defect during development, or are a secondary effect induced by obstruction due to abnormal secretions. CFTR mRNA has been localised to the testis of adult rats specifically within round spermatids and also within the principal cells lining the initial segment of the epididymis (Trezise *et al.*, 1993). In human new-born, infants and children CFTR has also been localised by *in situ* hybridisation to the epithelial cells within the head of the epididymis with an irregular distribution of signal in the epithelial cells of the corpus and cauda (Tizzano *et al.*, 1994). CFTR was also localised within the vas deferens. Tissue from adults displayed a similar pattern of expression. Even although all of the excurrent duct structures are derived from the mesonephric duct, the head of the epididymis (which in normal patients has the highest expression of CFTR) is the only region in which CFTR is consistently present in CF patients (Tizzano *et al.*, 1994). The lack of functional CFTR in the head of the CF epididymis may induce problems downstream due to flow obstruction. In the male, the sites of expression of CFTR have been identified but no studies have yet addressed its regulation. The existing evidence that CFTR is oestrogen regulated in the female reproductive tract, and that its peak expression coincides with the peak in uterine fluid volume, suggests that CFTR needs to be investigated to determine whether it is an oestrogen regulated gene in the male reproductive tract. This was the principal reason for investigating CFTR expression after neonatal oestrogen treatment in male rats.

There are also a number of similarities between the ion transport functions within the epididymis and those involved in urinary acidification within the kidney collecting duct. Similar ion transporter channels and mitochondrial rich cell types (which are associated with ion channel activity) are present in both organs. There are many features present in the transporting epithelial cells of vertebrates which have been used to group this cell type together as a family. These cells were originally referred to as mitochondria rich cells as they have a greater number of mitochondria than the surrounding epithelial cells (Brown and Breton, 1996). The major feature of these cells is that they express a vacuolar-type proton-pumping ATPase (H^+V-ATPase) within both intracellular vesicles and specific domains of the plasma membrane (Brown and Breton, 1996). The mitochondria rich cells of the kidney collecting duct are the intercalated cells. These cells contain a high level of carbonic anhydrase and H^+ATPase but virtually undetectable levels of Na^+/K^+-ATPase (Brown and

Breton, 1996). Mitochondria rich cells also possess apical microvilli and/or microplicae (broader plate-like extensions of the apical membrane), an active endocytotic pathway, as characterised by fluid phase endocytotic markers (i.e. horseradish peroxidase) and rod shaped intramembranous particles which are revealed during freeze fracture analysis (Brown and Breton, 1996).

The rat epididymis and vas deferens have both been shown to contain a subset of proton-pump rich cells which contain high levels of H^+ATPase on both their apical surface and in intracellular vesicles. These cells share all the characteristics of mitochondria rich cells. H^+ATPase has been localised to the apical or narrow cells in the caput epididymis and the clear cells in the corpus and cauda regions of the epididymis (Brown and Breton, 1996). The H^+ATPase can be specifically inhibited with bafilomycin and up to 80% of the transepithelial proton-flux can be inhibited within the vas deferens (Brown *et al.*, 1997). The apical and clear cells are thought to perform an analogous function to the intercalated cells of the collecting duct in that their primary function is in luminal proton secretion.

The studies presented in this chapter examined the state of the epididymal acidification machinery in peripubertal life, prior to the appearance of sperm within the epididymal lumen. A recent study has described the ontogeny of the H^+ATPase ion channel within the postnatal rat epididymis as determined using immunocytochemistry (Breton *et al.*, 1999). In the first week after birth, H^+ATPase immunostaining was very rare in all sections of the epididymis. The first staining in clear cells was evident during the second week after birth and by 3 weeks postnatal, epithelial cells immunostained for H^+ATPase were common in all epididymal regions. By week six it was evident that the number of H^+ATPase positive cells per millimetre of epididymis was significantly higher in the cauda epididymis than in the caput region and this pattern persisted into adulthood (Breton *et al.*, 1999). The increasing H^+ATPase activity in the distal regions of the epididymis supports its role in epididymal luminal acidification. The peak expression of H^+ATPase within the epididymis is reached before puberty and prior to the appearance of spermatozoa. This information has led researchers to argue that H^+ATPase-rich cells within the epididymis are not under the control of androgens (Breton *et al.*, 1999). Earlier studies have also indicated that maturation of the clear cells may not be driven by androgens (Hermo *et al.*, 1992), (Martinez *et al.*, 1995), but that the maturation and maintenance may be stimulated by oestrogens (Martinez *et al.*, 1995). Therefore the status of the H^+ATPase ion channels in the neonatally oestrogenised animals were an important target to check.

The studies in this chapter aimed to categorise changes in ion channel expression throughout the epididymis after neonatal oestrogen administration. The experimental work largely focuses on the expression of CFTR and ion channels involved in luminal acidification (H^+ATPase and the Na/H exchanger) but the expression of other ion channels will be mentioned where pertinent.

7.2 Materials and Methods

7.2.1 Treatments Regimes

This chapter focused on neonatal rats at day 25 after the administration of either GnRHa, DES (10µg/injection), ethinyl oestradiol or vehicle injected controls. For full details of the treatment protocols see Chapter 2 (Section 2.2).

7.2.2 CFTR *In Situ* Hybridisation

The *in situ* hybridisation protocol used in these studies was fully described in Chapter 2 (Sections 2.10 - 2.13). Two sets of primers were synthesised using the CFTR cDNA sequences in the published literature (Riordan *et al.*, 1989), (Fiedler *et al.*, 1992) and those entered into the GenBank database. The sequences chosen were

1. 5' CCT CAG CTG GAC CAC ACC ATT
   3' CGC TGA TTC CCA ACA ATA TGC

After PCR this synthesised an insert 787 base pairs long corresponding to nucleotides 184 and 977 at the 5' end of CFTR.

2. 5' CGA GGT GGG ATT CTT AAG AGA
   3' CAC AGA TCG CAT CAA TAT C

After PCR this synthesized an insert 576 base pairs long corresponding to nucleotides 3026 to 3602 at the 3' end of CFTR. Both primer sets produced bands of the correct size after PCR from tissue-specific cDNA pools of lung, pancreas or efferent ducts. However, the second set of primers produced a brighter set of bands and in subsequent PCRs only the second set of primers produced bands of the correct size. The CFTR insert which was initially amplified from the efferent duct cDNA was cloned into PCRII plasmid. Unfortunately, no specific signal was ever obtained using this insert as a labelled riboprobe with *in situ* hybridisation. Coincidentally, Dr P. Saunders performed studies on oestrogen regulated genes in the testis and identified a CFTR partial clone using differential display. Therefore this was used in future attempts at *in situ* hybridisation.

7.2.2.1 Identification of Ion Channels by PCR

To try to identify other ion channels involved in osmotic control within the excurrent duct system, primers were chosen to several ion channels (ATP-sensitive K^+channel, epithelial chloride channel, cGMP-gated cation channel and K-Cl Co-transporter) known to be expressed in other epithelial cell types, to determine if these were present in the excurrent ducts. Only the primers chosen for the ATPase sensitive K^+channel worked in PCR so only data obtained from this ion channel will be described. The methods involved in cDNA synthesis and PCR are fully explained in Chapter 2 (Sections 2.9 - 2.12). The primers used in this study were chosen by retrieving ion channel cDNA sequences from the GenBank database which were known to be expressed in epithelial cells. The primers chosen were

5' CTG TAC GAT ATC TCA GCC ACT

3' GTC TTA AGA GAC TCC TTC GCG

After PCR this represented a probe 322 base pairs long which started at nucleotide 1008 and ended at nucleotide 1330.

7.2.3 Fluorescent Immunocytochemistry

The tissue preparation and immunfluorescence protocols were described in Chapter 2 (Sections 2.3.1, 2.3.2, 2.4.2). The ion channels investigated were H^+ATPase (chicken polyclonal antibody raised against the 31KDa sub-unit of the H^+-ATPase) and the Na/H exchanger (rabbit polyclonal antibody raised to the Na^+/H^+ exchanger). This work was performed at the Renal Unit, Harvard Medical School, MA and the antibodies were provided by Prof. Dennis Brown and Dr Sylvie Breton.

These studies had to be completed within the 6 weeks of my visit to the Renal Unit and the data represent preliminary studies which were performed on only 3 animals from each treatment regimen, so all of the data presented must be regarded as preliminary. Due to the constraints of time all the digital images captured in these studies were compiled into montages in Edinburgh, and lack scale bars (Figures 7.3-7.5) as I did not have access to the appropriate lenses in order to add these to the figures.

7.2.3.1 Image analysis / Quantification

The number of H^+-ATPase positive cells in control and treated epididymides was quantified. Black and white photomicrographs were taken of the whole epididymis using a x20 objective. The final magnification of each image was x100. For each photograph the circumference of all tubules at the level of the basement membrane was determined using a Wacom graphics tablet and digitising pen connected to NIH image software, version 1.62

running on a Power Computing Power Tower Pro 225 computer. The software was calibrated to convert all measured lengths to millimetres. The total number of positive cells was counted for each photograph. These results were used to calculate the total number of stained cells found per millimetre of tubule circumference (cells/mm). A previous study by Breton et al., 1999 demonstrated that between weeks 1-4 postnatal there was no difference in the number of immunopositive cells between the caput and corpus epididymis (Breton *et al.*, 1999). As the data in this study is from animals at 25 days of age the data are not separated into caput and cauda epididymis.

7.2.3.2 Statistics

The number of positively stained H^+-ATPase cells within the epididymis was compared between control and treated cohorts using analysis of variance; where significant differences between groups were shown, subgroup comparisons were made using the same test, but the overall variance served as the measure of error.

7.3 Results

7.3.1 CFTR In situ hybridisation

These studies attempted to identify CFTR using radioactive *in situ* hybridisation in testes and excurrent duct samples neonatally oestrogen treated rats and controls. Uterus and adult testis samples were used as positive control tissues. No specific signal was ever detected in the neonatally oestrogenised or control tissue examined at day 18 or 25 using either of the riboprobes synthesised. However the probe generated from the differential display experiment (as described in Section 7.2.2) did produce a specific signal in both control tissues as illustrated in Figure 7.1.

Figure 7.1 shows four panels, with (a, b) illustrating the specific signal obtained with the antisense probe in adult testis and uterus respectively. Panels (c, d) demonstrate the non-specific signal obtained after incubation with the sense probe in testis and uterus respectively. Panel (a) shows a strong signal in the adult testis and clearly demonstrates that CFTR expression within the adult testis is stage specific and panel (b) shows a specific signal along the apical surface of the uterine epithelium although this signal is not as strong as that found within the testis. This is probably because the uterus sample was not staged and so was unlikely to be in pro-oestrus or oestrus which are the points in the oestrus cycle which display maximal CFTR expression.

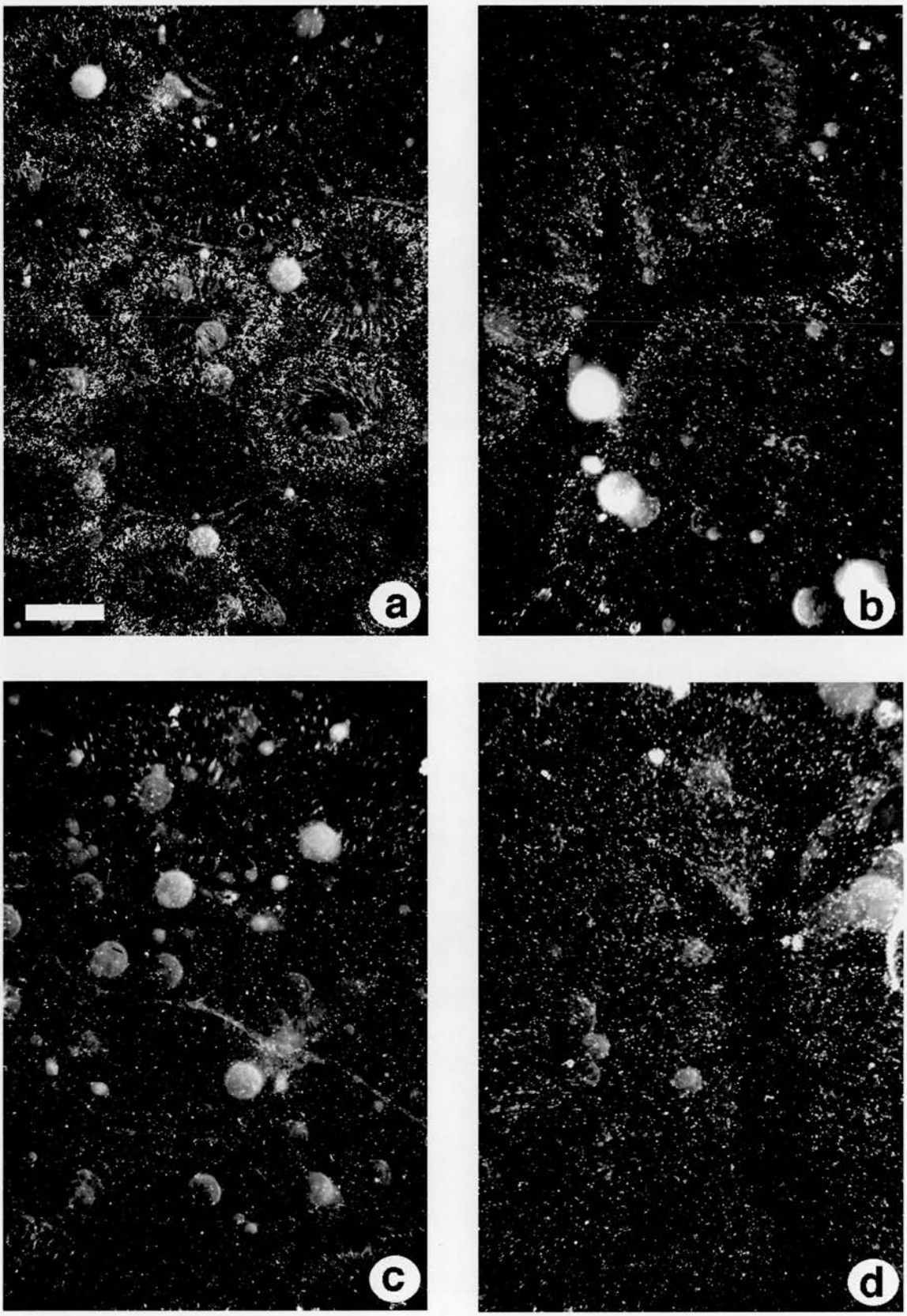

Figure 7.1 *In situ* **Hybridisation of CFTR in Adult Rat Testis and Uterus**
Illustrating the localisation of ^{35}S-UTP labelled CFTR riboprobe in (a, c) testis and (b, d) uterus. The antisense strands are shown in (a, b) and the sense strands in panels (c, d). The scale bar shows 150μm.

7.3.2 Tissue Distribution of ATP-sensitive K^+ Channel Detected by PCR

The tissue distribution of a ATP-sensitive K^+channel (which is known to be in epithelial cells of the heart (Hiraoka, 1997)) was checked with tissue specific cDNA libraries made from a broad panel of adult rat total RNA samples. Figure 7.2 illustrates an agarose gel displaying the tissue distribution of an ATP-sensitive K^+ channel with positive lanes showing bands corresponding with 322 base pairs.

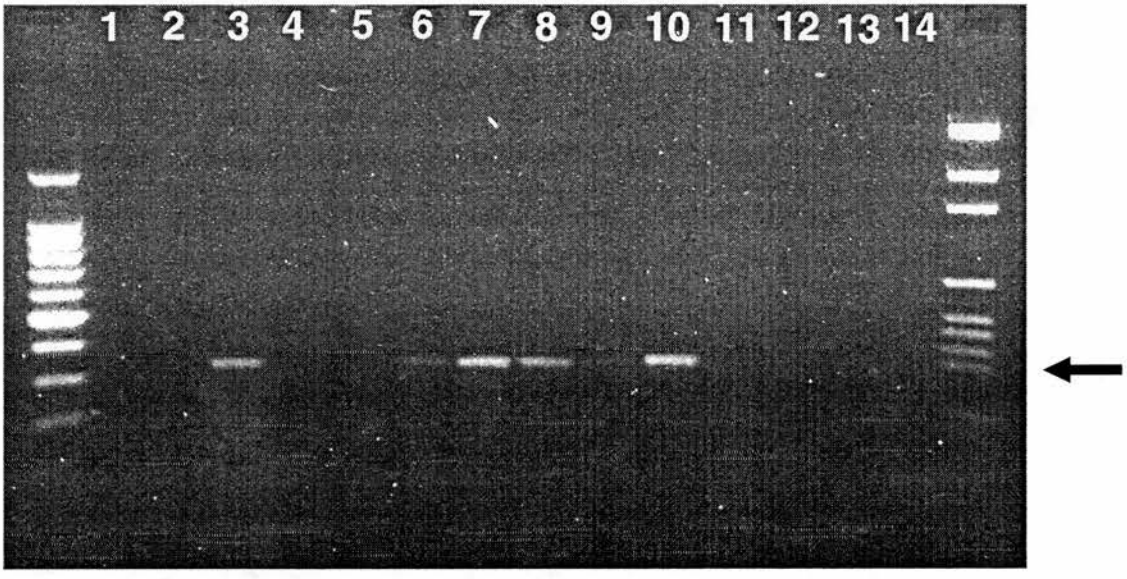

Figure 7.2 Distribution of ATP-Sensitive K^+ Channel in Adult Rat Tissue

Demonstrates the tissue distribution of a 322bp (arrow) section of an ATP-sensitive K^+ channel detected by PCR on an agarose gel containing 0.001% ethidium bromide. The samples in lanes 1-14 are as follows; control (lane 1), pancreas (lane 2), intestine (lane 3), brain (lane 4), caput epididymis (lane 5), lung (lane 6), heart (lane 7), efferent ducts (lane 8), testis (lane 9), ovary (lane 10), cauda epididymis (lane 11), prostate (lane 12), kidney (lane 13) and striated muscle (lane 14).

Figure 7.2 demonstrates that the ATP-sensitive K^+channel has a wide but specific tissue distribution. Strong bands were apparent in samples from intestine (lane 3), heart (lane 7), efferent ducts (lane 8), ovary (10) and faint bands were detectable in lung (lane 6) and testis (9) samples; very faint bands may also be present in the prostate (12) and kidney samples (13). No signal was detected in samples from the pancreas, brain, caput and cauda epididymis or muscle.

7.3.3 Immunofluoresence of H^+ATPase at Day 25 Postnatal

From previous studies on the postnatal localisation of H^+ATPase it is known to be expressed in the rat at its highest intensity at around 3 weeks postnatal (Breton *et al.*, 1999). In the present studies the expression of H^+ATPase was examined after neonatal treatment with either DES (10μg/injection), ethinyl oestradiol or GnRHa. As the data obtained after DES and ethinyl oestradiol treatments were essentially identical only photomicrographs of DES treatments will be shown. Figure 7.3 demonstrates both the alteration in the epididymal epithelium and the differences induced in H^+ATPase expression after neonatal treatment. Panel (a) demonstrates the intense immunostaining of H^+ATPase in the control epididymis. The staining was most intense along the apical surface of the epithelium but the whole of the apical cytoplasm was clearly strongly stained as were the lateral membranes. Panel (b) demonstrates the pattern of H^+ATPase immunofluorescence obtained after GnRHa treatment. It was clear that the number of positive cells was vastly reduced. The strong immunostaining along the apical cell surface was still evident on some cells but the cytoplasmic staining had largely disappeared. Similarly, panel (c) demonstrates the epididymal epithelium after neonatal DES treatment. Many cells showed a very thin apical band of H^+ATPase staining but, unlike the results for GnRHa treatment, there was very diffuse cytoplasmic staining which was not localised to the apical cytoplasm as it was in the control tissue, but appeared spread throughout all regions of the cell. It was also clear from these images that epithelial cells from the treated animals were grossly reduced in height.

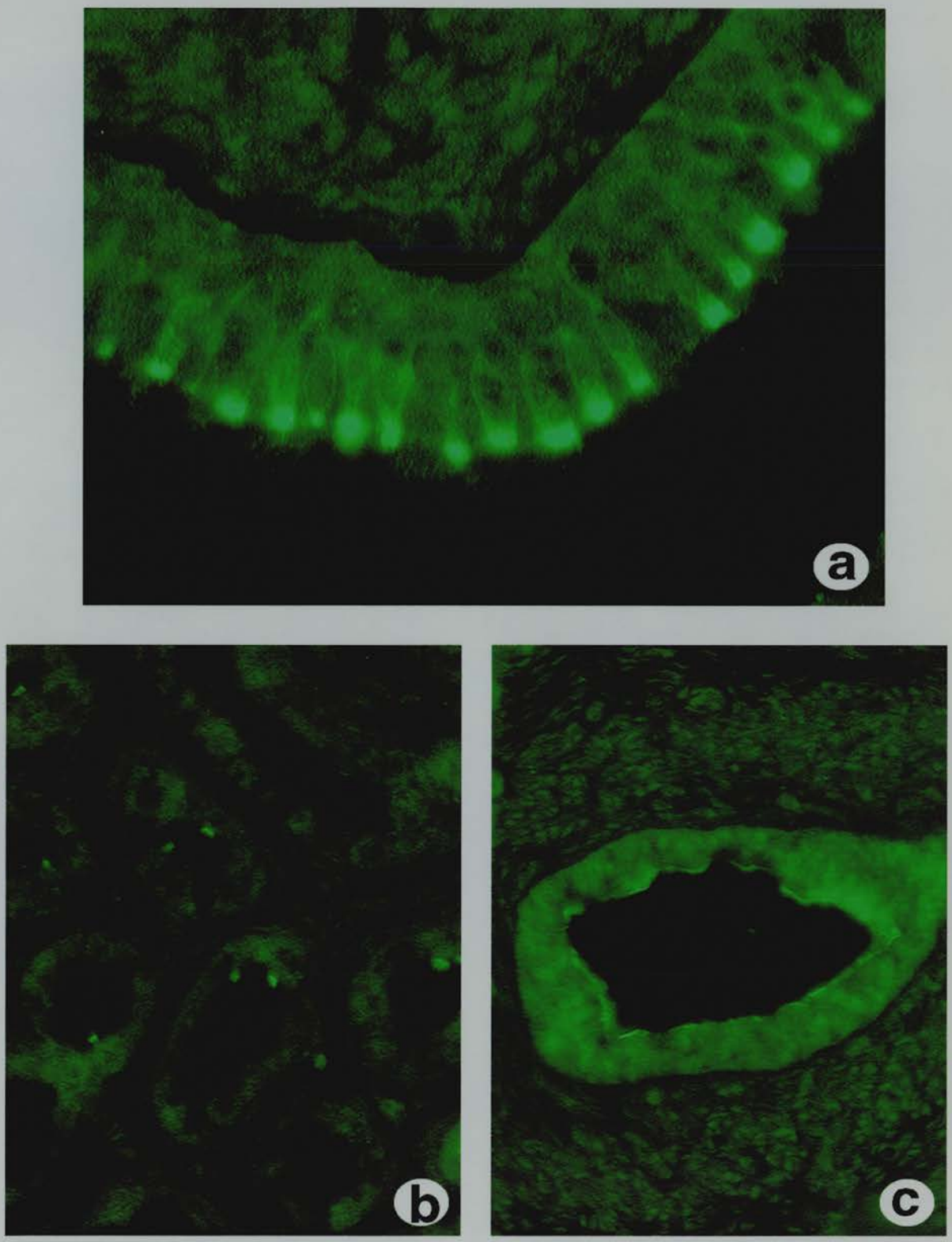

Figure 7.3 H^+-ATPase Immunofluoresence in Control and Treated Epididymides
Immunoexpression of H^+-ATPase in the epididymis of (a) control rat, or rats treated with (b) GnRHa, or (c) 10μg DES at day 25. Note the strong immunostaining of the apicalmembrane cytoplasm in (a) which is shown at a higher magnification to illustrate this feature.

7.3.3.1 Immunofluorescence of H^+ATPase and Na/H Exchanger at Day 25 Postnatal

Due to the profound morphological changes induced within the epididymal epithelium, it was necessary to identify a marker that had not changed in the epithelium to show that any changes were specific, and not a secondary effect induced by the malfunctioning of the whole organ due to neonatal oestrogen administration. It was found that the Na/H exchanger was not altered in expression in the epididymis of the Na/H exchanger and H^+ATPase visualised by fluorescence, with Figure 7.4 depicting a control animal while Figure 7.5 is from an animal treated neonatally with DES. In both figures panel (a) represents the merged image with both flurochromes present. The red staining represents the H^+ATPase and the green staining is the Na/H exchanger. Panel (b) illustrates the H^+ATPase staining only (red) and panel (c) shows only the Na/H exchanger (green).

Figure 7.4 clearly shows that both ion channel proteins are present in the control epididymis at day 25 postnatal. Figure 7.4 (panel (a)) illustrates that the majority of the cells appear to be double stained but others appear to have strong staining for only one of the ion channels. Figure 7.5 panel (a) is also double stained though it is evident that only the Na/H exchanger is present at any significant level in the neonatally treated DES epididymis. Panel (b) depicts the level of H^+ATPase ion channel staining and on careful examination a faint apical band of staining can be observed in some cells. Panel (c) shows a strong apical band of expression of Na/H exchanger though it is not as strong as in the control animal.

The various regions of the epididymis had different levels of ion channel staining, therefore in order to get a clear picture of the level of H^+ATPase ion channel staining present in the whole organ, the number of positive cells in the whole epididymis was counted. Due to the difference in the size of the epididymides induced after treatment, the circumference of each cross section of epididymis was measured and the number of H^+ATPase positive cells per millimetre of epididymal membrane was calculated. Figure 7.6 illustrates the number of H^+ATPase positive cells in animals treated with either GnRHa, DES (10μg/injection) or ethinyl oestradiol in comparison to control animals.

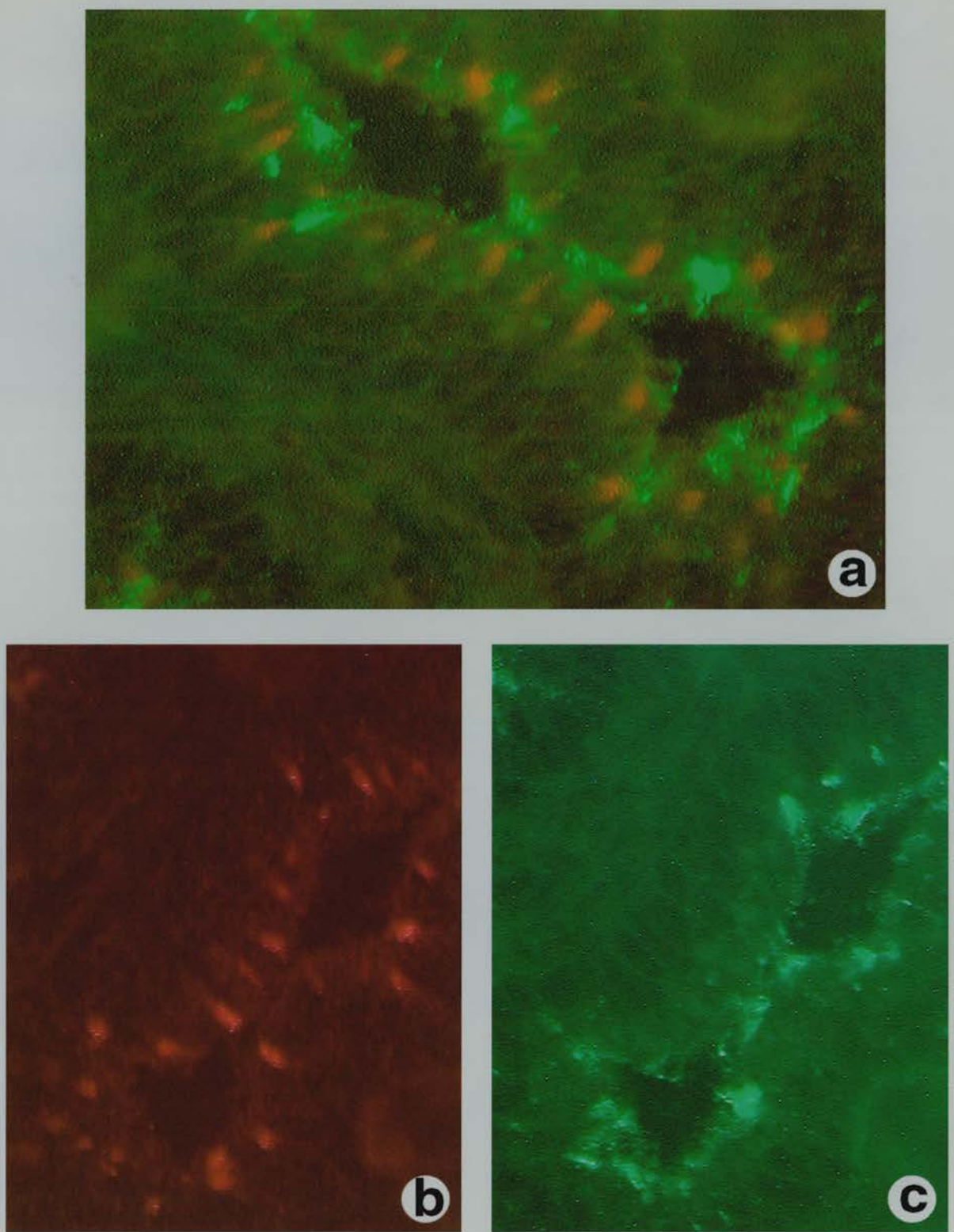

Figure 7.4 H^+-ATPase Immunofluoresence in Control Rat Epididymis
Immunoexpression of H^+-ATPase and Na/H exchanger in the epididymis of control animals at day 25. Panel (a) illustrates a double stained image with H^+-ATPase staining appearing in red and the Na/H exchanger localisation appearing green. Panels (b) and (c) illustrate the H^+-ATPase and Na/H exchanger single staining respectively. All panels were taken at the same magnification.

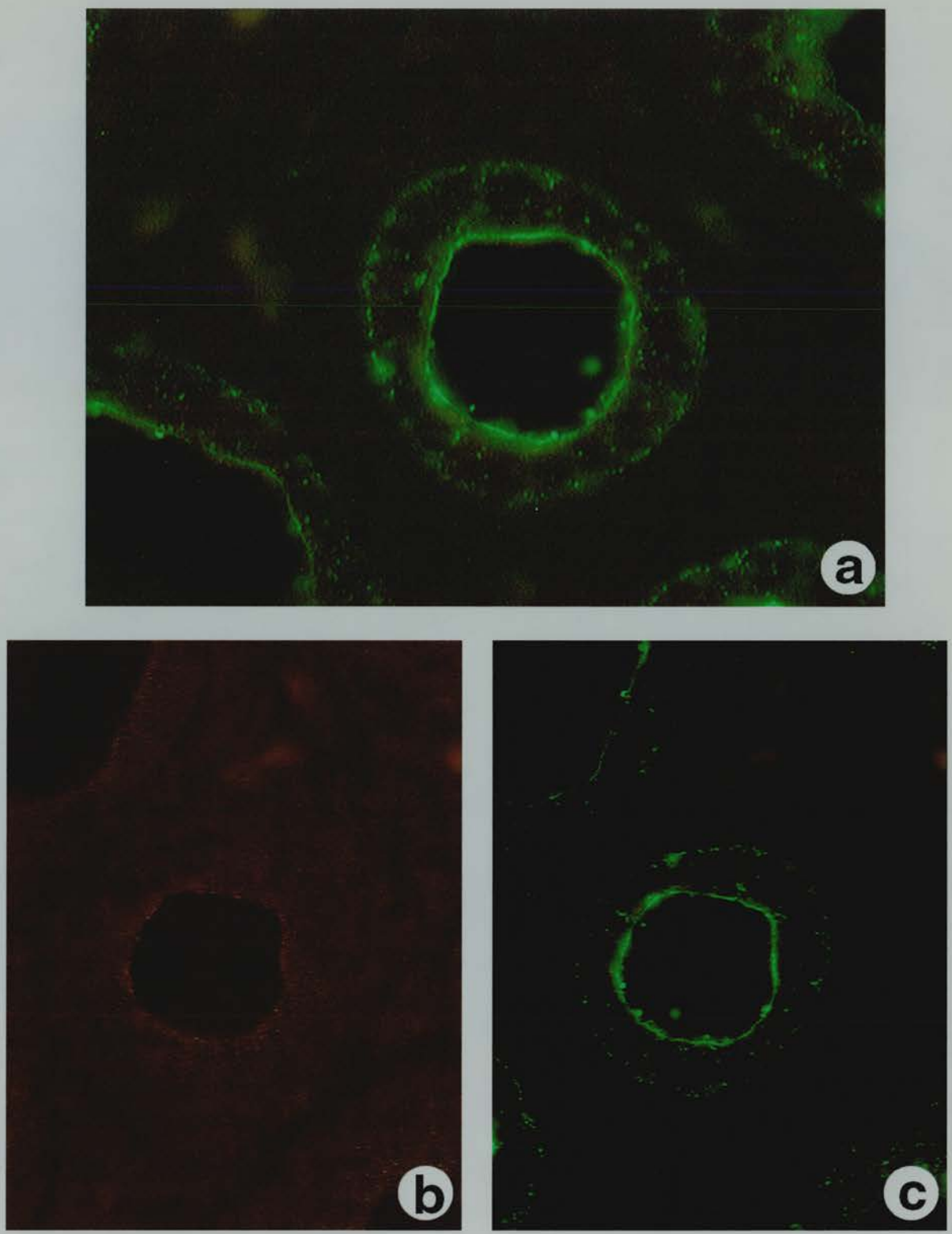

Figure 7.5 H^+-ATPase Immunofluoresence in DES Treated Rat Epididymis
Immunoexpression of H^+-ATPase and Na/H exchanger in the epididymis of a 25 day old animal treated neonatally with DES (10μg/injection). panel (a) illustrates a double stained image with H^+-ATPase staining appearing in red and the Na/H exchanger localisation appearing green. Panels (b) and (c) illustrate the H^+-ATPase and Na/H exchanger single staining respectively. All images were aquired at the same magnification.

The graph in Figure 7.6 is based on only 3 animals per treatment group. The control group had an average of 27.5 (±7.9) H^+ATPase-positive cells/mm of epididymal membrane. The large standard deviation observed in the GnRHa group (14.7±13.4 cells/mm) was due to one animal which had a level of H^+ATPase cell staining comparable with the control group, while the other two animals showed severe reductions in H^+ATPase cell staining. Both of the oestrogen treatments (10μg DES or ethinyl oestradiol) induced highly significant reductions in the number of H^+ATPase positive cells (2.44±1.59 and 5.86±8.15 respectively). The mean of the three ethinyl oestradiol treated animals also produced a large standard deviation. Again, was due to one ethinyl oestradiol treated animal which showed a far higher number of positive H^+ATPase cells/mm than did the other two animals, although the number of positive cells was still 50% of the control value. The results presented in Figure 7.6 require further investigation to determine whether the loss in H^+ATPase staining is due to a specific effect of oestrogen treatment or is a secondary effect induced by oestrogenic effects on the hypothalamic-pituitary axis.

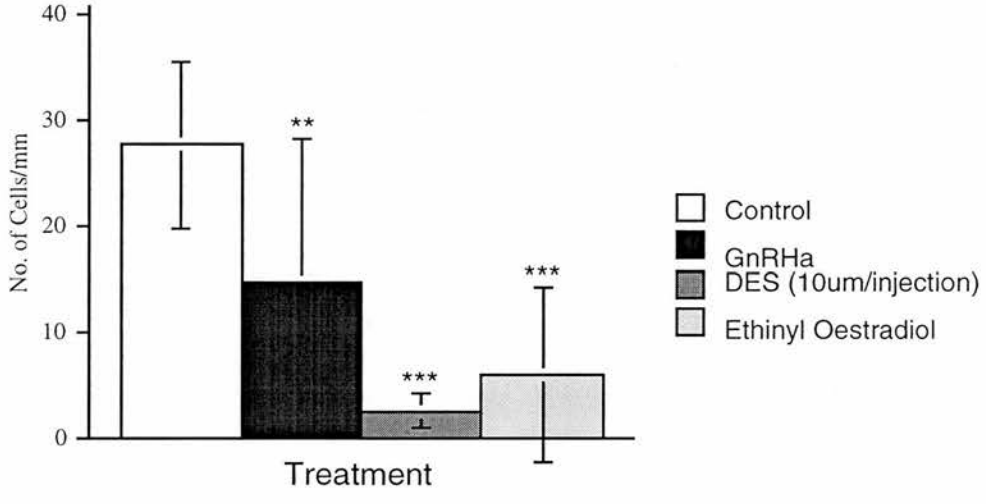

Figure 7.6 Comparison of the Number of H^+ATPase Cells in the Epididymis of Control and Treated Rats

The number of H^+ATPase cells/mm of epididymis were compared in 25 day old rats after neonatal treatment with vehicle (Control), GnRHa, DES (10μg/injection) or ethinyl oestradiol. (n= 3 for all groups; $^{**}p< 0.01$, $^{***}p< 0.001$ compared to control values)

7.4 Discussion

The studies presented in this chapter indicate that the mammalian excurrent duct system is a highly complex epithelium which expresses a multitude of ion channel proteins which are involved in the osmotic control and luminal acidification of the epididymis and its luminal contents.

Studies were performed to identify CFTR within the testis and excurrent ducts of control and neonatally oestrogenised testis and excurrent duct samples at 18 days of age. CFTR expression was identified by *in situ* hybridisation in control samples of adult testis and uterus, but no CFTR signal was detected in the postnatal control or neonatally oestrogenised samples. This may suggest that CFTR expression was not present in these tissues at day 18 postnatal. Or, it may be possible that a different splice variant of CFTR is expressed at this age which was not recognised by the probe used. Within the literature, the ontogeny of CFTR expression in the male reproductive tract has not been well mapped. In the rat testis, CFTR expression was first observed at day 10 postnatal and the hybridisation pattern was considered to be most representative of Sertoli cells (Trezise *et al.*, 1993). By day 28 postnatal CFTR expression was clearly evident in round spermatids (Trezise *et al.*, 1993). At postnatal day 10, CFTR expression is present throughout the proximal half of the epididymis, but a more restricted pattern of expression is observed by day 26 with CFTR expression resembling the adult pattern, with strong expression limited to the principal cells of the initial segment of the epididymis (Trezise *et al.*, 1993).

The lack of identification of CFTR in the postnatal samples is not due to the lack of expression of this mRNA. Time constraints did not allow an exhaustive study of this ion channel and it is important to determine whether or not CFTR is modulated by oestrogens in the male reproductive tract. Oestrogen regulated endpoints have to be identified to allow the oestrogenicity of 'environmental oestrogens' to be measured in an objective assay.

PCR studies attempted to identify other ion channels present with in the excurrent ducts. These studies successfully identified an ATPase sensitive K^+ channel in cDNA pools synthesised from efferent duct total RNA samples. This channel was found to be expressed in other tissues (heart, lung, intestine, ovary) but not in either the caput or cauda epididymis. ATPase sensitive K^+ channels are important in cardiac physiology and they are characterised by a strong inhibition by intracellular ATP (Hiraoka, 1997). ATP has a dual function in this channel, acting as a ligand which inhibits channel activity, however, it is also necessary for maintaining the channels in an operative state. During cardiac ischaemia the

opening of ATPase K^+ channels may contribute to the development of serious arhythmias but they also protect against cellular damage and improve recovery of cardiac function during reperfusion (Hiraoka, 1997). In the kidney, ATPase sensitive K^+ channels are present on the apical surface of the principal cells of the rat cortical collecting duct and their function is coupled to the basolateral Na^+/K^+ ATPase (Wang *et al.*, 1993). Na^+/K^+ ATPase has been localised to the efferent duct epithelium (Byers and Graham, 1990) raising the possibility that a similar relationship may also exist in this organ. Further experiments are required to confirm the expression of ATPase sensitive K^+ channels in the efferent ducts of the male reproductive tract.

The final studies described in this chapter involved the expression of two ion channel proteins involved in epididymal luminal acidification. A low bicarbonate concentration and an acidic pH in the luminal fluid of the epididymis are important for sperm maturation. Two proton excretion proteins, an Na^+/H^+ exchanger and an H^+ATPase have been proposed to be involved in this process (Jensen *et al.*, 1999). H^+ATPase is known to be expressed on the apical membrane of apical (narrow) and clear cells of the but the Na^+/H^+ exchanger has yet to be described in situ (Jensen *et al.*, 1999). This study illustrated the effect of neonatal oestrogen treatment on the expression of these two ion channels in the epididymis of 25 day old postnatal rats.

Figure 7.3 clearly demonstrated the change in H^+ATPase immunostaining detected after neonatal treatment with GnRHa or DES (10 μg/injection). The GnRHa treated animals show a clear reduction in epididymal H^+ATPase immunostaining, but intense apical staining could clearly be observed. In contrast, DES treatment induce a vast reduction in the number of cells showing clear apical staining. Positive cells showed a very thin band of apical staining unlike the thick intense staining observed in the apical cells of the control epididymis. Another stark change in H^+ATPase immunostaining after neonatal DES (10μg/injection) treatment was the diffuse cytoplasm staining which was not apparent in control animals.

The vacuolar H^+ATPase is structurally related to the F(1)F(0) ATP synthases of mitochondria, chloroplasts and bacteria (Xu *et al.*, 1999). The mammalian V-type H^+ATPases are composed of two subunits, a peripheral V1 and an integral V0 domains. The V1 domain is a 570 KDa peripheral complex composed of 8 subunits (A-H) which is responsible for ATP hydrolysis and the V0 domain is a 260 KDa integral complex composed of 5 subunits which are responsible for proton translocation (Forgae, 1998).

Aside from functions in luminal acidification in the epididymis and urinary acidification the kidney, H^+ATPases are also involved in receptor-mediated endocytosis, intracellular membrane trafficking, protein degradation as well as coupled transport (Forgae, 1998).

In the kidney and liver, microtubules are involved in the rapid and directed movement of ion channels and transporters to and from the apical plasma membranes (Hamm-Alvarez and Sheetz, 1998). Given the similarities between kidney and excurrent duct physiology, it is probable that microtubules are involved in the insertion and removal of H^+ATPases into and from the apical membrane. The change from primarily apical (in control rats) to diffuse cytoplasmic staining observed in the DES treated cohort could be accounted for by a displacement of the H^+ATPase containing vesicles from the apical cytoplasm to other regions of the cell. This could either be induced by disordered microtubule formation or an alteration of signal peptides which prevent the molecules from being directed to their correct cellular location.

These studies suggest that the distribution of ion channel proteins within the male excurrent duct system is likely to be complex with each specific region of the duct expressing different proteins. These proteins alter the luminal environment of the developing spermatozoa but what regulates the expression of these protein remains to be determined. The relative roles of the steroid hormones, peptide hormones or the spermatozoa themselves in determining the pattern of protein expression remain an issue which has to be resolved before the roles of a specific factor i.e. oestrogens or oestrogenic compounds can be determined with certainty.

Chapter 8 General Discussion

The objectives of the work conducted for this thesis were several-fold:

- To determine sites of oestrogen receptor expression within the excurrent ducts of the male reproductive tract.
- To identify the direct effects of neonatal oestrogen exposure on the excurrent ducts
- To investigate functional markers of the excurrent duct system which could be modulated by oestrogen, monitored throughout neonatal and adult life and that could be used to assess the relative oestrogenicity of so called 'environmental oestrogens'.

Firstly, the excurrent ducts were examined as recent studies suggested that the efferent ducts have the highest expression of ERα within the male reproductive tract; secondly, studies on ERKO mice (which are infertile) show fluid accumulation within the rete testis and excurrent ducts and, finally, the characterisation of a second oestrogen receptor (ERβ), suggested that there were even more targets for oestrogen action within the male reproductive tract. The effects of neonatal oestrogen exposure were studied due to the current concerns over human male reproductive health. The literature currently suggests that the sperm count is falling with later year of birth, and there is evidence to suggest increases in the incidence of testicular cancer, cryptorchidism and hypospadias (Toppari *et al.*, 1996). These conditions were also shown to be increased in prevalence in the sons of mothers administered DES during pregnancy. These similarities led to the hypothesis that oestrogenic chemicals in the environment could be involved in the increases in disorders of the male reproductive tract (Sharpe and Skakkebaek, 1993). There are now many reports in the literature regarding chemicals, which are widespread in the environment, and which have been found to be weakly oestrogenic. Whether weakly oestrogenic chemicals pose any real risk to reproductive health is a hotly debated issue.

These studies were performed to determine the effects of neonatal administration of potent oestrogens (DES and ethinyl oestradiol) on the male excurrent ducts and to determine whether similar effects could be induced after exposure to weakly oestrogenic 'environmental oestrogens'.

8.1 The Sites of Oestrogen Receptor Expression Within the Male Reproductive Tract

Chapter 3 examined the sites of ERβ expression within the marmoset testis and excurrent ducts from postnatal day 1 to adulthood. The expression of ERβ was compared to those of ERα and the AR in the adult marmoset testis and excurrent ducts. The results are summarised below

1. ERβ imunoexpression in the testis and excurrent ducts of the marmoset

- •The adult pattern of immunostaining was present by 8 weeks of age. At this age there was immunoexpression in Sertoli cells, spermatogonia, peritubular myoid cells and interstitial cells of the testis
- •Similarly, within the efferent ducts, nuclear immunostaining was present in both the ciliated and nonciliated cells but not all cell nuclei were stained.
- • At 8 weeks postnatal, nuclear immunostaining was present in the principal, basal and apical cells of the caput epididymis, and again this was comparable to the adult expression pattern.

2. Comparison of ER (α and β) and the AR in the testis and excurrent ducts of the marmoset revealed that:

- • All three steroid receptors displayed a different cellular expression pattern within the testis and excurrent ducts, but of the three, ERβ had the widest cellular distribution.

- Within the testis all three steroid receptors were localised to Leydig cells, only ERβ and the AR were localised to Sertoli cells but ERβ was the only steroid receptor to be localised to gonocytes and the developing spermatogonia.
- All three steroid receptors were localised to the efferent ducts and all were evident within both ciliated and nonciliated cells. All three had a checker-board pattern of expression. Only ERβ was localised within the surrounding stromal tissue.
- ERα did not localise to any cell types within the epididymal epithelium but ERβ and the AR displayed similar sites of immunolocalisation to principal, basal and apical cells.

These studies demonstrated that there were overlapping sites of AR, ERα and ERβ within the testis and male reproductive tract. All three steroid receptors were expressed within the Leydig cells of the testis and the epithelial cells of the efferent ducts. The checker-board pattern of immunostaining observed within the efferent ducts (that was common to all three steroid receptors) raised the question as to whether the cells were only expressing one of the steroid hormone receptors, hence the reason why the other cells were not immunopositive. To address this issue, double or even triple immunofluorescence could have been performed in order to determine whether these steroid receptors were being expressed in discrete cells or whether all of the steroid receptors were expressed together in the same cell. Similarly, in the epididymis ERβ and AR shared a very similar expression pattern i.e. within the same cell types. This suggests that there may be interplay between the actions of oestrogens and androgens within these cells. The exact nature of the interplay between androgens and oestrogens within the male reproductive tract is not understood. The recent localisation of aromatase to developing germ cells (Nitta *et al.*, 1993) suggests that the conversion of androgen to oestrogen can occur within the testis and during the transit of sperm through the efferent ducts and epididymis,

therefore the balance of androgen to oestrogen may be determined by the local environment.

These studies were important initial steps to clarify the sites within the excurrent ducts that oestrogens could be expected to act and they also heightened awareness of the potential existence of a locally controlled androgen to oestrogen balance in the excurrent ducts. Therefore subsequent studies focussed on the excurrent ducts when examining the effects of neonatal oestrogen treatment.

8.2 Morphological Changes Induced in the Rete Testis and Efferent Ducts After Neonatal Oestrogen Exposure in the Rat

The experiments conducted in this study were performed on animals after neonatal treatment with potent oestrogens (DES or ethinyl oestradiol). The treatment was originally developed to coincide with the period of Sertoli cell multiplication. Animals were assessed at days 18 and 25 postnatal (around the initiation of puberty), day 35 (late puberty) and day 75 (early adulthood). A cohort of animals was also assessed at day 10 while the animals were still within the treatment phase.

Morphological changes observed in the rete testis and efferent ducts of rats treated neonatally with oestrogens were discussed in Chapter 5 and 6. The major morphological findings are summarised below:

- The lumens of the rete testis and efferent ducts were grossly distended after exposure to DES (10μg and 1.0μg) or ethinyl oestradiol.
- Rete testis distension was evident (to a lesser degree) in adult life, while efferent duct distension was not readily detected after day 35 postnatal.
- Epithelial cell height was grossly reduced in the rete testis, efferent ducts and the caput epididymis after neonatal exposure to DES and ethinyl oestradiol. Epithelial

cell height was measured in the efferent ducts and was found to be reduced dose-dependently by DES.

- Weakly oestrogenic compounds also induced small but significant reductions in efferent duct epithelial cell height (except parabens).
- No significant reduction in epithelial cell height was detected in any treatment group by adulthood.

The reason why neonatal oestrogen exposure induced morphological changes in cell shape and height or why treatment induced fluid accumulation within the rete testis and efferent ducts is not known. All of these changes were concluded to be direct effects of oestrogen as none of them were observed in animals treated neonatally with a GnRHa, a treatment that suppressed gonadotrophin secretion, and thus testicular development, to a comparable degree to that induced by treatment with 10μg DES.

In these studies, distension and fluid accumulation were evident as early as postnatal day 10 in both the rete testis and efferent ducts, which raised the question as to the source of the fluid. In adult animals, STF is secreted by the Sertoli cell and forms the fluid which enters the excurrent duct system. The Sertoli cells of the adult testis form a barrier via intercellular tight junctions, which creates a separate luminal compartment within the seminiferous tubules. This barrier enables a unique microenvironment to be created and maintained in the adluminal compartment that is different from that in the interstitium. However, in early neonatal life, neither the Sertoli cell barrier nor seminiferous tubule lumens have developed (i.e. tight junction formation has not yet occurred) therefore the source of the fluid which accumulates after neonatal oestrogen exposure is not obvious. The most likely source of fluid is the Sertoli cell but the lack of barrier and lumen formation mean that fluid flow is difficult to detect as fluid is free to diffuse into the testis from the interstitial fluid. Fluid may also enter the excurrent duct system via some other

source i.e. the lymphatic system. In order to determine if the fluid is flowing via the testis or an extra testicular source, efferent duct ligation could be performed on neonatal animals. If the efferent ducts remain patent after ligation, this would suggest an extratesticular source for the fluid accumulation but if the fluid dilation was more severe behind the ligature this would suggest a testicular source for the fluid.

Changes in epithelial cell height were observed in the rete testis, efferent ducts and epididymis but this change were only quantified in the efferent ducts. DES and ethinyl oestradiol induced gross reductions in epithelial cell height, whereas weakly oestrogenic compounds induced small but significant reductions. However, no significant decrease in epithelial cell height was detected in adulthood after any of the treatments. The regulation of epithelial cell height within the reproductive tract is not understood. However, a tall, columnar appearance is a characteristic of the differentiated cell (Raczek *et al.*, 1994). The lack or loss of this characteristic suggests that neonatal oestrogen administration delays or disrupts the differentiation of this cell type. The alteration is, however, not permanent. The exact pathway involved in epithelial cell differentiation is not known but is bound to be complex and so determining whether this pathway could be disrupted by neonatal oestrogen exposure will not be simple.

Cell and organ culture experiments suggest that androgens are important for maintaining cell height *in vitro* (Tezon and Blaquier, 1981),(Vazquez *et al.*, 1989). However, no studies have assessed the influence of oestrogen on epithelial cell height. Epithelial cell height is reduced in the male ERKO mouse which has elevated serum testosterone levels, suggesting that testosterone is not the only factor involved. A more likely explanation may come from an alteration of the underlying basal lamina. The basal lamina is a mat of specialised extra cellularmatrix which underlies all epithelial sheets and tubes. Aside from a structural role, the basal lamina can influence cell polarity, cell metabolism, the

organisation of proteins in the adjacent plasma membrane and it can induce cell differentiation (Alberts *et al.*, 1994). As the basal lamina is largely secreted by the epithelial cells on which it rests, any alteration in its secretion and subsequent functions could disrupt the normal function and/or morphology of the epithelial cells. Whether oestrogen can alter the composition or function of the basal lamina, is unknown, but this could be examined using *in vitro* studies. However, all epididymal cell and organ cultures have reduced cell height suggesting that there are many factors involved in regulating cell height and the normal functioning of epithelial cells.

The changes in cell height and rete testis/efferent duct distension always occurred in parallel and with similar severity which was dose-dependent. This makes it difficult to determine cause and effect. The most logical interpretation for the distension and reduction in cell height is that neonatal oestrogen exposure induced a disruption in endocytosis. Such a disruption would prevent the resorption of fluid within the efferent ducts leading to their dilation and subsequent backflow and dilation within the rete testis. To investigate this studies could be performed using electron dense tracers such as cationic ferritin to determine whether the endocytoyic pathway is disrupted.

8.3 Functional Changes Within the Excurrent Ducts After Neonatal Oestrogen Treatment

Functional changes within the rete testis, efferent ducts and epididymis were assessed in Chapters 4, 6 and 7. The major strategy involved determining the major function of this tissue (fluid resorption) and investigating likely candidate proteins from the homologous kidney structure, the proximal tubule. This strategy suggested that a water channel protein originally termed CHIP28 and subsequently renamed AQP-1, was a good candidate molecule. A previously published report demonstrated that AQP-1 was expressed by the adult efferent duct epithelium. An antibody was kindly gifted by Prof.

Dennis Brown and was used initially to determine if AQP-1 was expressed in the efferent ducts during neonatal life. AQP-1 was found to be expressed from fetal life, throughout neonatal and, pubertal life and into adulthood. Within the rat, this protein was localised to the efferent ducts and was not expressed in the testis or epididymis. As the efferent ducts are a major site of ERα expression it was not inconceivable to suggest that AQP-1 could be modulated by oestrogen. However, AQP-1 was located along the apical and basolateral membranes of all non-ciliated cells and was not present along the membranes of ciliated cells. In contrast, ERα is expressed in both cell types although not all cell nuclei expressed the receptor at a given time.

The immunoexpression of AQP-1 was assessed after neonatal treatment with potent oestrogens (DES and ethinyl oestradiol) and the weakly oestrogenic 'environmental oestrogens'. These studies demonstrated that neonatal exposure to 10μg DES or ethinyl oestradiol induced almost a complete loss of AQP-1 immunostaining in animals aged 18 and 25 days of age. This reduction in protein levels was corroborated by Western Blotting. Subjective analysis of AQP-1 immunostaining suggested that DES dose-dependently reduced the level of immunostaining when assessed in 18 and 25 day old animals, but by adulthood the immunostaining levels were indistinguishable from control animals. Some of the animals treated with weakly oestrogenic compounds consistently displayed a slight reduction in the intensity of immunostaining but this was not an objective assessment and only gross changes could be detected with certainty. The promoter region of the AQP-1 gene does not contain any consensus EREs but does contain an AP-1 site which can activate target genes after binding the proto-oncogenes c*Fos* and c*Jun*. This suggests that there is the potential for oestrogen to regulate AQP-1 gene transcription.

However, the reduction in AQP-1 did not occur in isolation but was observed in parallel with the changes in cell height and fluid distension suggesting that neonatal oestrogen treatment is unlikely to be responsible for regulating the expression of a single gene such as AQP-1. If oestrogen directly induced the loss of AQP-1 then the lack of fluid resorption might induce distension, but this is unlikely as water resorption can also occur secondary to ion transport in this epithelium. But even if a loss of AQP-1 did induce fluid accumulation through a lack of fluid resorption this could still not necessarily account for the loss of cell height observed after neonatal oestrogen treatment.

The overall impression from these changes was that oestrogen has control over a central cellular process which could account for the range of effects observed instead of just one aspect of them. The type of changes observed after neonatal oestrogen treatment (loss of cell height, possible disruption of the endocytotic pathway, loss of proteins from the apical membrane), and supportive data from the literature, suggest that the most probable site of oestrogen action is on the cytoskeleton. Oestrogen has been shown to have nongenomic effects on microtubules. Oestrogen administration can induce a rapid loss of microtubules in endometrial cells within 80 seconds and which returns to control levels after 30mins but then re-occurs after 1 hour (Szego *et al.*, 1988). Microtubules are critical for both the maintenance of cell polarity and membrane trafficking. Disrupting microtubule formation in kidney proximal tubule cells induces a breakdown in endocytosis as no endocytotic invaginations or large vacuoles are formed (Elkjaer *et al.*, 1995). In this study, disrupting microtubule formation also reduced the level of membrane recycling and induced almost a complete loss of AQP-1 immunolocalisation. A similar explanation would account for the loss of AQP-1 immunostaining from the apical membrane of epithelial cells in the efferent ducts, the fluid accumulation via a disruption of endocytosis and the loss in cell height due to the lack of formation of the apical tubules and other structures involved in the endocytotic pathway which comprise a

major proportion of the apical cytoplasm. Studies need to be performed to determine whether this is a viable hypothesis. Studies of oestrogen action on cell structure may be feasible by short-term organ culture experiments. This would allow assessment of the effect of oestrogen administration on the membrane architecture, and microtubule formation. Knowing the speed at which any changes occurred would help to differentiate genomic and non-genomic effects.

Similarly, within the epididymis the preliminary data presented in Chapter 7 suggested that the number of H^+ATPase positive cells/mm epididymis was reduced after neonatal exposure to DES and ethinyl oestradiol. However, there was also a significant reduction in H^+ATPase staining after GnRHa administration though this reduction was not as severe as that induced after either DES or ethinyl oestradiol administration. A larger sample size is required to determine whether this decrease in cell staining is an effect of oestrogen acting indirectly by altering gonadotrophin secretion. However, both GnRHa and 10μg DES treatments produced different immunostaining patterns for H^+ATPase. The GnRHa still induced a bright apical band of H^+ATPase although the intensity was much reduced compared to control animals. After DES treatment, there were very few cells which displayed any apical membrane staining. There were no particular areas of intense staining but instead there was diffuse staining throughout the cytoplasm. The diffuse H^+ATPase staining suggests that vesicles are not being directed to the apical membrane and underlying cytoplasm. If this were the explanation, such an alteration in H^+ATPase distribution could only be induced by alteration to the cytoskeleton and studies in kidney and liver have shown that microtubules are involved in the insertion and removal of H^+ATPases to and from the plasma membrane.

In summary the studies presented in this thesis have demonstrated that potent oestrogens can have a detrimental effect on the postnatal development and functioning of the

excurrent duct system. In particular, the normal fluid dynamics of this system were disrupted as evidenced by the loss of AQP-1 immunostaining, the dilation of both the rete testis and efferent duct lumens and the alterations to efferent duct epithelial cell morphology. These findings could be best explained by changes to the epithelial cell cytoskeleton, although the studies performed in this thesis did not address this possibility. In contrast, weakly oestrogenic compounds did not induce gross changes in fluid dynamics and only minor changes in AQP-1 immunostaining and efferent duct epithelial cell height. Within the controlled laboratory environment these chemicals had little effect on the male reproductive tract when administered at extremely high doses (not relevant to man) and at what is considered the most sensitive time-point. However, the consequences of chronic exposure of animals to a combination of these weak oestrogens (as occurs in reality) was not assessed, and more research needs to be performed to assess whether realistic mixtures of these compounds pose any risk to human health.

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Appendix 1

Appendix 1 Table comparing the bodyweight of animals at necropsy after the neonatal administration of various compounds

Treatment	Day 18	Day 25	Day 35	Day 75
Control	41.3±5.7 (n=24)	56.4±5.6 (n=8)	143.0±5.0 (n=6)	453.3±138.3 (n=6)
GnRHa	36.4±7.1[b] (n=12)	62.9±7.2 (n=8)	110.3±2.5[a] (n=3)	431.4±48.9 (n=5)
DES 10μg	26.1±1.6[a] (n=14)	58.9±10.2 (n=8)	119.6±4.7[a] (n=5)	402.3±54.9 (n=4)
DES 1μg	46.4±5.2[b] (n=10)	66.6±3.6[c] (n=5)	121.4±11.8[a] (n=5)	477.0±36.2 (n=5)
DES 0.1μg	44.1±1.9 (n=7)	52.0±3.7 (n=4)	109.5±11.8[a] (n=4)	450.0±23.9 (n=4)
Ethinyl Oestradiol	32.7±3.8[a] (n=4)	50.5±13.3 (n=7)	-	464.33±3.8 (n=3)
Genistein	44.1±4.6 (n=14)	78.9±8.1[a] (n=7)	-	606.4±32.1[a] (n=5)
Octylphenol	40.9±1.9 (n=8)	55.3±1.8 (n=6)	-	-
Bisphenol A	41.2±4.6 (n=6)	55.4±9.6 (n=5)	115.0±6.9[a] (n=3)	520.3±45.1 (n=3)
Parabens	41.7±2.7 (n=6)	-	-	

Key : [a] $p<0.001$, [b] $p<0.01$, [c] $p<0.05$

Appendix 2

Publications Arising From This Thesis

Fisher, JS; Turner, KJ; Brown, D; Sharpe, RM
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Co-Author Publications

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